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NYMPHAEA L. GENUS IN THE DANUBE DELTA, ROMÂNIA

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Abstract: This paper is focused on the two species of the *Nymphaea* genus, present in the flora of the Danube Delta, respectively *Nymphaea alba* L. and *N. candida* C. Presl. The paper has several objectives as follows: i) to illustrate the morphological identification features mentioned in the literature for *Nymphaea alba* and *N.candida*, using material collected from the Danube Delta, ii) to complete the existing information with anatomical-histological data regarding the structure of the lamina and the petiole, with emphasis on the particularities of the mechanical, assimilating and conducting tissues of the two hydrophytes, iii) to present and characterize a variety of the taxon *Nymphaea candida* – *Nymphaea candida* var. *undulatifolia* var. *nova*, identified in 2012, in the Danube Delta on the Bărbos Canal and the Letea Canal, with a confirmed presence in the period 2012-2015, 2017-2019.

Keywords: anatomy, aquatic plants, identification features, morphology, new taxa.

Introduction

Nymphaeaceae are aquatic dicotyledons, rarely amphibians [SĂVULESCU (ed.), 1952-1976], well represented in the warm climatic areas in the world.

According to the specialised literature [CIOCÂRLAN, 1994; CIOCÂRLAN & al. 1998; CIOCÂRLAN, 2009; SÂRBU & al. 2013; SĂVULESCU (ed.), 1952-1976], Fam. *Nymphaceae* Salisb., is represented in the Flora of Romania by: *Nelumbo* Adams. (naturalized plants), *Nuphar* Sm. and *Nymphaea* L. (spontaneous plants). The *Nymphaea* genus includes three species: *Nymphaea lotus* var. *thermalis* (DC.) Tuzs., *Nymphaea alba* L. and *Nymphaea candida* J. Presl. Rostl. Out of these, two can be found in the Danube Delta: *Nymphaea alba* and *Nymphaea candida* [CIOCÂRLAN, 2009; SÂRBU, 2006; SÂRBU & al. 2011; SÂRBU & al. 2013; SÂRBU, 2015]. For *Nymphaea alba* it is mentioned in the Danube Delta and the variety *minoriflora* (Barb.) A. et G. Syn. [SĂVULESCU (ed.), 1952-1976].

Nymphaea alba and *Nymphaea candida* are perennial, rooted hydrophytes with rhizome, large floating leaves, long petioles and white flowers, which stand above the water, supported by long floral peduncles. The same characteristics define the plants of *Nymphaea alba* var. *minoriflora*, except for the fact that they are shorter and the leaves and flowers are smaller compared to the size of the type [SĂVULESCU (ed.), 1952-1976].

Nymphaea alba differs from *Nymphaea candida* in terms of morphological characteristics [MUNTENDAM & al. 1996]. In this sense, the data from the literature document the taxonomic utility of a wide range of morphological identification characters: nervation, shape of the flower base, shape of internal stamens, shape and color of the stigma, number of lobes of the stigma, shape of the gynoecium in longitudinal section, shape and pollen architecture, fruit shape [KABÁTOVÁ & al. 2014; MUNTENDAM & al. 1996; NOWAK & al. 2010; SÂRBU, 2015; SÂRBU & al. 2013; VOLKOVA & SHIPUNOV, 2007; WATSONA & DALLWITZ, 1992].

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Despite being based on the same organization plan, the histo-anatomical structure of the vegetative body of *Nymphaea alba* and *Nymphaea candida* plants [SCHNEIDER & WILLIAMSON, 1993; STRASBURGER & al. 1999] present some differences, often dimensional or quantitative, which address especially the lamina, petiole and floral peduncle [SÂRBU & al. 2014; TOMONIN, 2017; WATSON & DALLWITZ, 1992].

The ecological preferences of the two species of *Nymphaea* have some peculiarities in relation to the acidity and trophicity of the water: i) *Nymphaea alba* prefers both alkaline and weakly acidic waters [MUNTENDAM et al. 1996, SÂRBU et al. 2018] and is eutrophic tolerant [KABÁTOVÁ & al. 2014], ii) *Nymphaea candida* prefers alkaline [MUNTENDAM & al. 1996], mesotrophic and eutrophic waters with a high content of organic matter [NOWAK & al. 2010]. Both species grow in stagnant or very smoothly flowing, not very deep waters (0.5-2 m), and *Nymphaea candida* prefers waters near shore areas [NOWAK & al. 2010]. In the Danube Delta *Nymphaea alba* and *Nymphaea candida* populate the shallow, eutrophic waters of canals, lakes, ponds and swamps [SÂRBU & al. 2013].

This paper addresses the *Nymphaea* species in the Danube Delta and aims to:

i. illustrate the morphological identification characters for *Nymphaea alba* and *Nymphaea candida*, using material collected from the Danube Delta;
ii. complete the existing information with anatomical-histological data, referring to the organization of the lamina and the petiole;
iii. present and characterize a new variety of the *Nymphaea candida* taxon from a morpho-anatomical point of view: *Nymphaea candida* var. *undulatifolia*.

Material and methods

The biological material, represented by mature, well-developed plants belonging to the *Nymphaea* genus, was collected from the Danube Delta (Lake Bodgaproste, Magearu Canal, Bărbos Canal, Letea Canal), during 2012-2015 and 2017-2019, respectively.

The identification of the 2 species of *Nymphaea* (*Nymphaea alba* and *Nymphaea candida*) was performed *in situ* and was based on the use of morphological identification criteria, mentioned in the literature. The parameters concerned the characteristics of the leaf and the flower: the shape and dimensions of the lamina, the leaf rib, the shape of the flower base, the shape of the flower stalk, the shape of the gynoecium in longitudinal section, the characteristics of the filaments of the internal stamens, the number of lobes of the stigma.

Some of these morphological parameters were also used to describe two varieties, one (*Nymphaea alba* var. *minoriflora*), associated with the *Nymphaea alba* species and cited in the literature as being present in the Danube Delta [SĂVULESCU (ed.), 1952-1976] and the other (*Nymphaea candida* var. *undulatifolia*) associated with the *Nymphaea candida* species and described for the first time in this paper.

Anatomo-histological observations concerned the leaf (lamina and petiole) and were performed on ten specimens of *Nymphaea alba* and *Nymphaea candida*, respectively, and on five specimens of *Nymphaea candida* var. *undulatifolia*. Manually cross sections were made in the central part of the lamina on the median nervure, in the marginal part of the lamina and through the petiole, in the median third. Differential and successive colorations of crossed material with Iodine Green and Carmine Alum have been applied [ŞERBĂNESCU-JITARIU & al. 1983]. All microscopic slides have been analysed with a DOCUVAL optical microscope in normal lights. Photomicrographs have been obtained with a microscope incorporated Nikon D90 digital camera.

Results

1. *Nymphaea alba* and *Nymphaea candida* – morphological identification features

Leaf. In the two *Nymphaea* species, the mature leaves differ morphologically (Plate I, Figure 1; Plate II, Figure 1). In *Nymphaea alba*, the blade is ovate to almost subround (Plate I, Figure 5), ~30 cm long and ~26 cm wide (Table 1). On the lower face of the lamina the nerves are obvious and anastomosed.

In *Nymphaea candida*, the lamina has an ovate shape, longer than wide (~28 cm long and ~19 cm wide) (Table 1). The nervures are prominent on the lower face and the first pair at the base is curved (Plate II, Figure 5). The petiole is cylindrical and with 40% thicker in *Nymphaea alba* compared to *Nymphaea candida* (Table 2).

The base of the flower. The shape of the flower base is different in the two *Nymphaea* species: the base is rounded in *Nymphaea alba* and obtuse 4-edged in *Nymphaea candida* (Plate I, Figure 2; Plate II, Figure 2; Table 3).

The flower stalk. In cross section the floral stalk is round in *Nymphaea alba* while in *Nymphaea candida*, it has several rounded edges (Plate II, Figure 6).

The shape of the stigma. The longitudinal sectioning of the flower, allows to analyze the shape of the stigma. It is flat in shape in *Nymphaea alba* and obviously concave in *Nymphaea candida* (Plate I, Figure 3; Plate II, Figure 3).

The lobes of the stigma. The number of lobes of the stigma reflects the number of carpels that participated in the formation of the gynoecium. This number is different for the two *Nymphaea* species (Table 3): stigma with 14-23 lobes on the edge in *Nymphaea alba* and stigma with 8-14 lobes on the edge in *Nymphaea candida* (Table 3).

Filaments of internal stamens. In *Nymphaea alba*, the filaments of the internal stamens are narrower and narrower, reaching the width of the anthers. These filaments either have the same width along their entire length or are very slightly wider in their median area (Plate I, Figure 4). In *Nymphaea candida*, the filaments of the internal stamens are wider than the anthers and have the maximum width in the middle (Plate II, Figure 4).

2. *Nymphaea alba* and *Nymphaea candida* – comparative structural aspects

The histo-anatomical observations made on the two *Nymphaea* species concerned the leaf (lamina and petiole), a vegetative organ accessible throughout the vegetation period of these plants.

Lamina. The structure of the lamina was analyzed, on cross sections made in the central part of the lamina (on the median nervure and outside it) and in the marginal part of the lamina.

In both plants, the lamina has the characteristic structure of the *Nymphaea* genus. The lamina is bifacial heterofacial, the mesophile showing a dorsi-ventral organization of the assimilating tissues (adaxial palisadic and abaxial lacunar tissue). The lacunar tissue draws an aerenchyma with polygonal air ducts in the area of the large nervures and with wide canals in the rest of the mesophile. The air ducts are delimited by partition, lamellar, uniseriate walls. The mechanical elements are represented by columnar sclereids with branched top, located in the palisade tissue, by longer or shorter astrosclerids, more or less branched, present in the lacunare tissue and by solitary filiform idioblasts, which often accompany the subepidermal angular collenchyma. The main nervure is prominent at the abaxial face of the lamina, is often accompanied by areas of angular collenchyma and

contains several collateral conducting bundles, devoid of vascular cambium. The same type of conducting bundles is found in the secondary nervures of different orders, which pass through the mesophile.

The observations made on the histo-anatomical organization of the lamina of *Nymphaea alba* and *Nymphaea candida* respectively, highlighted a series of differences, especially from a quantitative point of view (Table 1). In *Nymphaea alba*, in the central part of the lamina, the median nervure is ~20% thicker and the mesophilic ~10% more voluminous, compared to *Nymphaea candida* (Plate III, Figures 1-4). Palisade tissue is approximately the same thickness in both species (250.0 μm in *Nymphaea alba* and 260.0 μm in *Nymphaea candida)*. In *Nymphaea alba*, the palisade tissue represents ~38% of the mesophilic thickness, and in *Nymphaea candida* ~44%. The lacunar tissue occupies 62% of the mesophilic thickness in *Nymphaea alba* and only 56% in *Nymphaea candida* (Table 1, Plate III, Figure 3, Figure 4). In both species, the mesophilus is about 23-24% thinner at the edge of the lamina than the middle of the lamina. Palisade columnar sclereids are ~10% longer and ~40% thicker in *Nymphaea candida* than in *Nymphaea alba* (Table 1, Plate III, Figures 5-6).

Petiole. In cross section, the petiole highlights a circular shape. The petiole of *Nymphaea alba* is thicker (1.5 cm in diameter) compared to the petiole of *Nymphaea candida* (0.9 cm in diameter).

The histo-anatomical structure of the petiole is similar in the two species and respects the general organization plan for the *Nymphaea* genus.

Under the unilayered epidermis, in the multilayered angular collenchyma are present filiform sclereids (rod-shaped sclereids), arranged equidistantly, in series. In the fundamental parenchyma of the petiole, the large, medium and small air ducts are arranged relatively symmetrically.

The vascular system is represented by a modified eustel [SCHNEIDER & WILLIAMSON, 1993]. The conducting bundles are collateral, single or double and show no vascular cambium. The xylem is represented by few elements and the protoxylematic gap is present. Both single and double conducting bundles were observed in both species. In *Nymphaea candida* the conducting bundles are 25-30% more numerous, but their dimensions are slightly smaller (Table 2, Plate IV, Figure 5, Figure 6).

In the petiole of *Nymphaea alba*, the subepidermal angular collenchyma includes 7-8 layers of cells and has an average thickness of 250.0 μm (Table 1, Plate IV, Figure 3). In *Nymphaea candida*, the area of the collenchyma is ~30% thicker and includes 9-10 layers of cells (Table 1, Plate I, Figure 4). Both *Nymphaea alba* and *Nymphaea candida* have large, medium and small air ducts in the structure of the petiole. Both species have 4 large air ducts (1800.0 μm diameter *Nymphaea alba* and 1500.0 μm *Nymphaea candida*) located in the center of the petiole. Medium-sized air ducts (800.0 μm diameter) of the petiole of *Nymphaea candida* are ~ 35% more numerous compared the number of average air ducts (900.0 μm diameter) observed in *Nymphaea alba* (Table 2, Plate IV, Figure 1, Figure 2).

Diaphragmatic tissue was observed in both species, located especially in small but also medium air ducts (Plate IV, Figure 7, Figure 8).

3. *Nymphaea alba* var. *minoriflora* - morphology

Nymphaea alba var. *minoriflora* (Barb.) A. et G. Syn. is mentioned as being present in the Danube Delta by SĂVULESCU (ed.) (1952-1976) in the work Flora României. In the period 2012-2015 and 2017-2019, this variety of the *Nymphaea alba*

species was observed on the Letea Canal. The plants are short (60-70 cm long), with smaller leaves and flowers than those of *Nymphaea alba* (Plate V, Figure 1). The leaves have an almost subround blade, with the entire edge (Plate V, Figure 2). The base of the flower is round (Plate V, Figure 3), and the stigma is flat (Plate V, Figure 4), characters which brings it closer to *Nymphaea alba*.

4. *Nymphaea candida* var. *undulatifolia* – morphology and structure

Nymphaea candida var. *undulatifolia* var. *nova* (Foliis minoribus constat ex typ differt a marginibus obvious undulatis et in floribus minoribus) was identified (Sârbu I.) in the Danube Delta in August 2012, on the Bărbos Canal and on the Letea Canal, at the edge of *Nymphaea candida* populations (Plate VI, Table 1-3). The presence of the infrataxon was reconfirmed in 2013-2015 and 2017-2019, respectively (Sârbu A.). The nomenclatural type is in the Herbarium of the Botanical Garden "Dimitrie Brândză" in the University of Bucharest, registered with the number 404475.

The plant is a perennial rooted, with a rhizome, with long petiolate floating leaves, a thin, entire, broad-ovate lamina with a wavy edge (Plate VI, Figure 1, Figure 6). The lamina is much smaller (15 cm long and 13 cm wide) than that of *Nymphaea candida* and the petiole is ~30% thinner (Table 1, Plate VI, Figure 2). The flowers are smaller than those of *Nymphaea candida*, they are white, 6-6.5 cm in diameter and the flower bud is only 3.8 cm long (Table 3, Plate VI, Figure 4). The base of the flower is obtuse 4-edged and the stigma is concave as in *Nymphaea candida*, (Plate VI, Figure 3, Figure 5) with 8-16 lobes, respectively. The filaments of the internal stamens are wider than the anthers, as in *Nymphaea candida*.

The structure of the lamina corresponds to the *Nymphaea* genus. The median nervure is prominent on the adaxial face and almost as thick as in *Nymphaea candida* (Table 2, Plate VII, Figure 1). The mesophil is 40% thinner at the edge of the lamina, compared to its central part, and the palisadic tissue represents ~50% of the mesophilic thickness (Table 1, Plate VII, Figure 2). Palisadic columnar sclereids are shorter than those of *Nymphaea candida*, but vigorous, especially those at the edge of the lamina (45.0 μm thick) (Table 1, Plate VII, Figure 2, Figure 3). The much thinner edge of the lamina and the presence of a smaller number of columnar sclereids at the top of the lamina could determine to some extent, the undulation of this area, on the surface of the water (Table 1, Plate VII, Figure 2, Figure 3).

The petiole is circular in cross section and highlights all the characteristic elements of a *Nymphaea* petiole. However, a series of structural features bring it closer to the petiole of *Nymphaea candida*: the thickness of the subepidermal collenchyma (Table 2, Plate VII, Figure 5), the number of single and double closed collateral type conducting bundles (Table 2), the number of medium-sized air ducts (Table 2, Plate VII, Figure 4).

In this variety, some of the smaller air ducts (200.0-300.0 μm in diameter) are delimited by a layer of larger cells, whose internal tangential walls and lateral walls are obviously thickened (Plate VII, Figure 4, Figure 6).

Table 1. Analyzed lamina parameters (in cross section): *Nymphaea alba*, *N. candida*, *N. candida* var. *undulatifolia*.

Parameters		Measurements/size (average values)		
		Nymphaea alba	***Nymphaea candida***	***Nymphaea candida* var. *undulatifolia***
Dimension of the lamina	Length (cm)	30.0	28.0	15.0
	Width (cm)	26.0	19.0	13.0
The central part of the lamina	Median rib thickness (μm)	2500.0	2000.0	2200.0
	Rib collenchyma thickness (μm)	570.0	460.0	450.0
	Mesophil thickness (μm)	650.0	580.0	500.0
	Palisadic tissue thickness (μm)	250.0	260.0	270.0
	Lacuna tissue thickness (μm)	400.0	320.0	230.0
	Sclereids length (μm)	450.0	500.0	260.0
	Sclereids thickness (μm)	23.0	38.0	27.0
The marginal part of the lamina	Mesophil thickness (μm)	500.0	450.0	230.0
	Sclereids length (μm)	400.0	350.0	220.0
	Sclereids thickness (μm)	23.0	38.0	45.0

Table 2. Analyzed petiole parameters (in cross section): *Nymphaea alba*, *N. candida*, *N. candida* var. *undulatifolia*.

Parameters		Measurements/size (average values)		
		Nymphaea alba	***Nymphaea candida***	***Nymphaea candida* var. *undulatifolia***
Diameter of the petiol (cm)		1.5	0.9	0.6
Collenchyma thickness (μm)		250.0	350.0	320.0
Number of collenchyma layers		7.0 - 8.0	9.0 - 10.0	8.0 - 9.0
Double conducting bundles	Number	8.0	10.0	8.0
	Length (μm)	1300.0	900.0	500.0
Simple conducting bundles	Number	10.0	14.0	12.0
	Length (μm)	550.0	450.0	200.0
Large air ducts	Number	4.0	4.0	4.0
	Diameter (μm)	1800.0	1500.0	800.0
Medium air ducts	Number	10.0	14.0	12.0
	Diameter (μm)	900.0	800.0	600.0

Table 3. Analyzed flower parameters: *Nymphaea alba*, *N. candida*, *N. candida* var. *undulatifolia*.

Parameters	Measurements/size (average values)		
	Nymphaea alba	***Nymphaea candida***	***Nymphaea candida* var. *undulatifolia***
Length of the floral bud (cm)	9.0	7.0	3.8
Flower diameter (cm)	12.0 - 13.0	10.0 - 11.0	6.0 - 6.5
Flower base	rounded shape	4-edged shape	4-edged shape
Stigma shape	flat shape	concave	concave
Number of the stigma lobes	14.0 - 23.0	8.0 - 14.0	8.0 - 16.0
Filaments of internal stamens	narrower or as wide as the anthers	wider than the anthers	wider than the anthers

Conclusions

The paper exemplifies a set of morphological identification features for *Nymphaea alba* and *Nymphaea candida*, scientifically accepted and usable in their in situ identification. The use of plant material collected from the Danube Delta, confirms the existence of both taxa in the Danube Delta.

The two species assessed, *Nymphaea alba* and *Nymphaea candida*, differ from each other in some structural aspects. These are predominantly dimensional and/or quantitative and target in particular mechanical tissues, assimilative and conductive tissues.

The paper updates the presence of the *Nymphaea alba* var. *minoriflora* taxon in the Danube Delta and documents the observations, with current photographic images.

The paper describes a new infrataxon – *Nymphaea candida* var. *undulatifolia* var. *nova*, identified in the Danube Delta and his morphological and structural characteristics, which support its classification on the variety position of the *Nymphaea candida* taxon.

Based on research conducted in 2012-2019 in the field and in the laboratory, the paper confirms the presence of two species of the *Nymphaea* genus, namely *Nymphaea alba* and *Nymphaea candida* in the Danube Delta and of two varieties of these species: *Nymphaea alba* var. *minoriflora* and *Nymphaea candida* var. *undulatifolia*.

Notes on contributors

Anca SÂRBU – is a university professor with a special interest in plant anatomy, flora and vegetation, evaluation and conservation of phytodiversity. She carried out cyto-histological and morpho-anatomical research on protected plants in the Romanian flora, research on aquatic plants in the Danube and Danube Delta, research on plant diversity in Natura 2000 habitat types.

Ion SÂRBU – is a research scientist with a special experience in the plants taxonomy, chorology (vascular plants), phytosociology and habitats. His research focused on the flora and vegetation of Romania, the identification of Natura 2000 sites in Romania, the characterization of the habitat types and plant communities from the most diverse areas of botanical importance in Romania.

Anca-Monica PARASCHIV – is a plant biologist, interested in the study of spontaneous and cultivated plants. She contributed to the development of morpho-anatomical research on some cultivated plants with medicinal value and on some vascular plants from the Danube Delta.

Clara Daniela MIHAI – is a plant biologist, interested in the study of spontaneous plants with emphasis on their anatomy and on the associated histo-anatomical techniques. She was involved in morpho-anatomical researches addressed to spontaneous plant from Bucegi Mountain and Danube Delta.

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Plates explanations

PLATE I *Nymphaea alba* – morphology

Fig. 1. *Nymphaea alba* in situ
Fig. 2. Flower base
Fig. 3. Flat stigma
Fig. 4. Filaments of the internal stamens
Fig. 5. Lamina

PLATE II *Nymphaea candida* – morphology

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Fig. 3. Concave stigma
Fig. 4. Filaments of the internal stamens
Fig. 5. Lamina
Fig. 6. Floral stalk

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Fig. 3. Flower base
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Fig. 5. Petiole, subepidermic collenchyma (1 – epidermis, 2 – angular collenchyma)
Fig. 6. Petiole, air channel delimited by cells with thickened walls (1 – air duct)

Plate I

***Nymphaea alba* – morphology**

Figure 1

Figure 2

Figure 3

Figure 5

Figure 4

Plate II

***Nymphaea candida* - morphology**

Figure 1

Figure 2

Figure 3

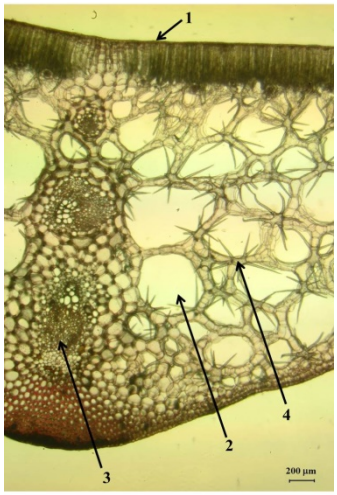
Figure 4

Figure 5

Figure 6

Plate III

***Nymphaea alba* & *Nymphaea candida* – Lamina structure**



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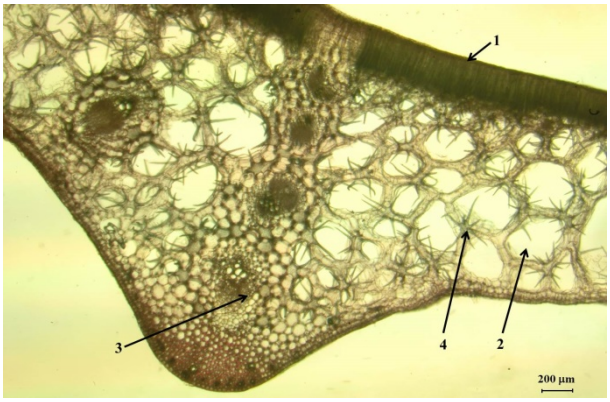

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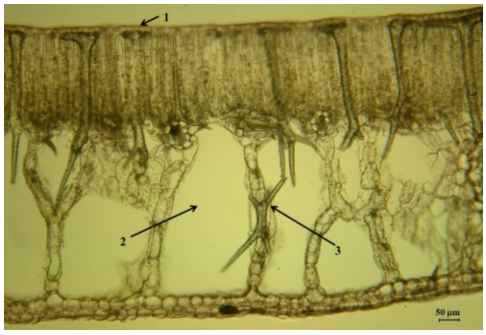

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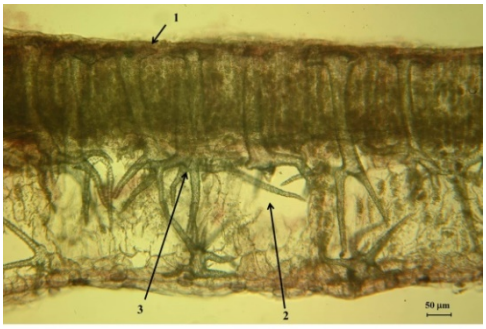

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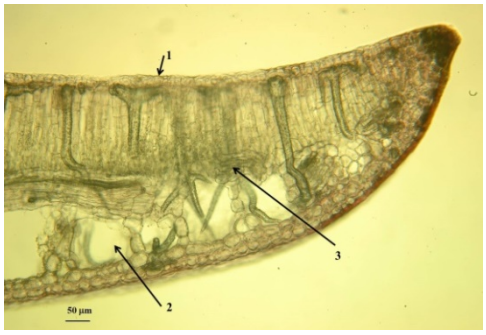

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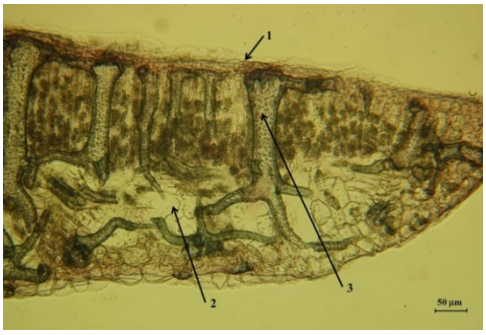


Figure 6

Plate IV

***Nymphaea alba* & *Nymphaea candida* – Petiole structure**

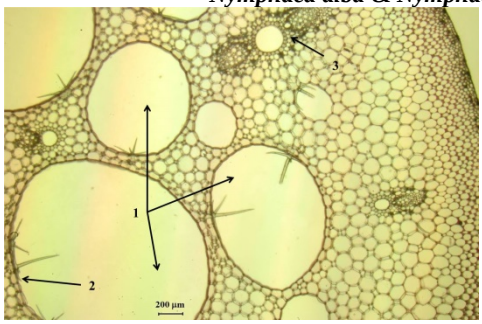
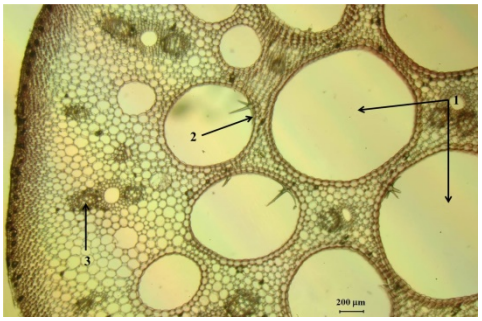


Figure 1



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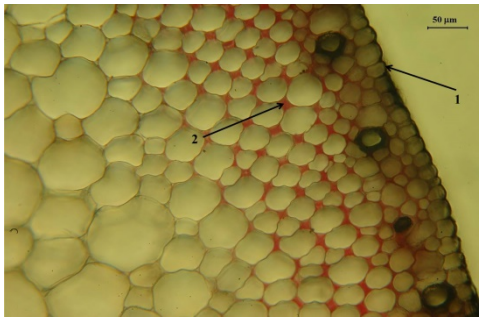

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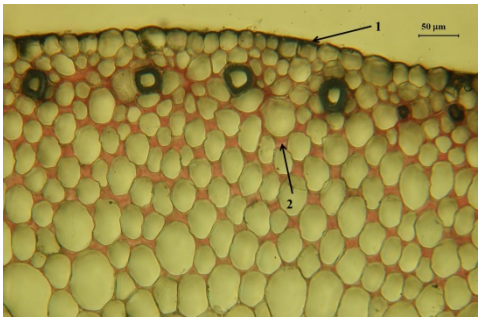

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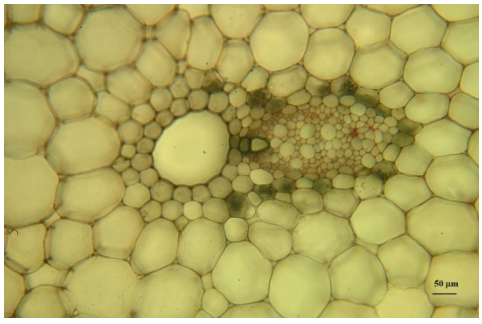

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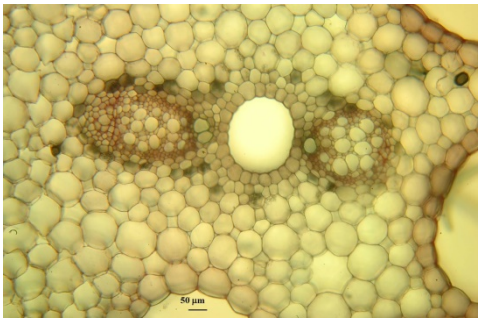

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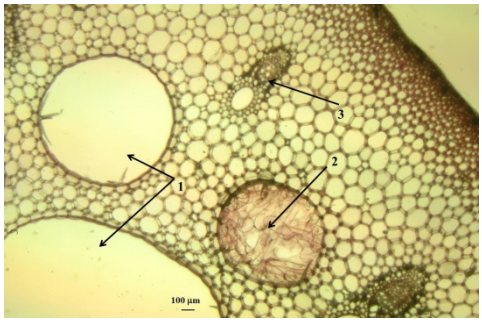

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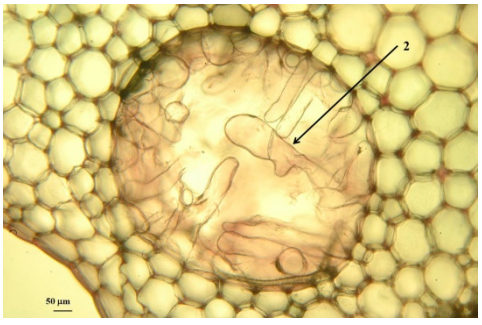


Figure 8

Plate V

***Nymphaea alba* var. *minoriflora* – morphology**

Figure 1

Figure 2

Figure 3

Figure 4

Plate VI

***Nymphaea candida* var. *undulatifolia* – morphology**

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Plate VII

***Nymphaea candida* var. *undulatifolia* – Lamina and petiole structure**

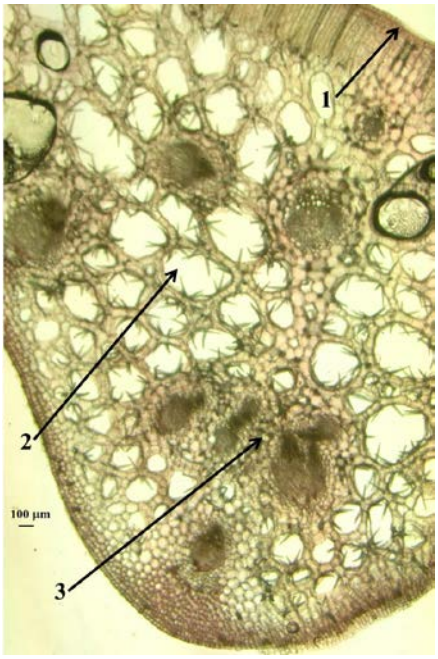
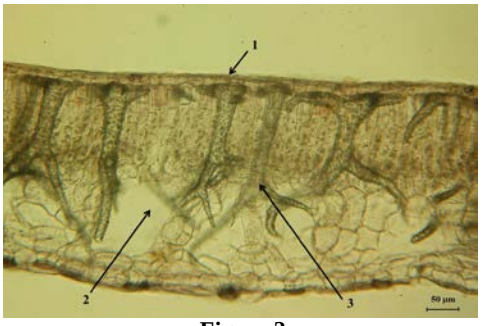


Figure 1



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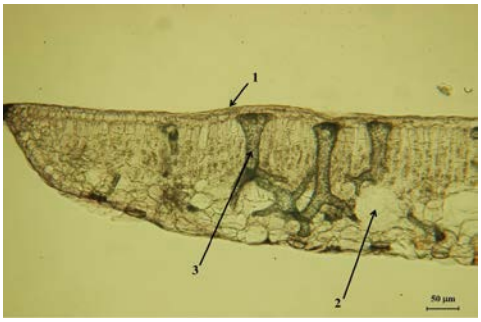

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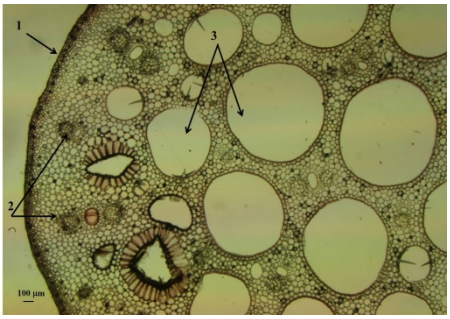

Figure 4

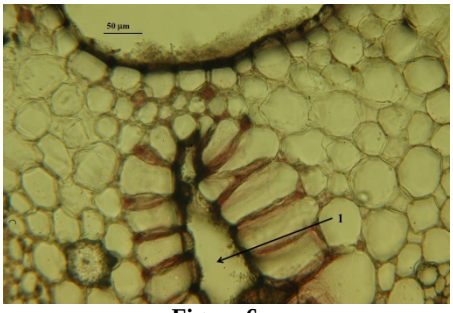

Figure 6

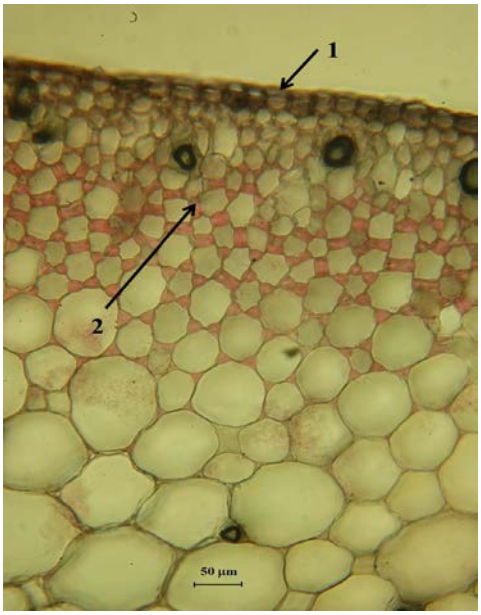

Figure 5

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TISSUE DIFFERENTIATION OF THE EARLY AND THE LATE FLOWER BUDS OF *RAFFLESIA PATMA* BLUME

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Abstract: The flower bud of *Rafflesia patma* Blume following the protocorm stage of the flower will undergo rapid cell growth followed by the differentiation stage which lead to the later stage of the flower bud morphogenesis into the floral organs. During this transitional period of the flower bud, we revisited our *R. patma* flower bud microscope slide samples and some images from the previous study in [MURSIDAWATI & SUNARYO, 2012] to examine how the *R. patma* tissue behave prior to the late differentiation stage. We discovered that there are several types of meristematic cells in the *R. patma* early flower bud tissue: The elongated cells in the basal/proximal region of the bud where it in proximity with host xylem, then a column of non-elongated cells where ovary will develop in the later stage (in female flower), and in the apical/distal region of the flower bud, we found a densely packed meristematic cells where in the later flower bud this area will be developed into the protective bracts, perigone lobes, and central disc as later seen in the late flower bud tissue. During the late stage of the flower bud growth, the flower bud also inhibits growth of 1-2 vascular bundles, altering few others host vascular bundles surrounding the flower bud, while on the other side the root vascular bundle growth is just normal. This growth mechanism can minimize the host vasculature damage if multiple buds are growing the same growth direction.

Keywords: histology, holoparasite, parasitic plant, plant development, Rafflesiaceae.

Introduction

Rafflesia growth within its host is shrouded with mysteries. In previous studies, the developmental origin of the organ has been observed by NIKOLOV & al. (2013) using cloning of the RNA isolation from MADS-Box genes (B-class lineages *PISTILLATA [PI]* and *APETALA3 [AP3]*, and C/D-class genes *AGAMOUS [AG]* and *SEEDSTICK [STK]*) using *Rafflesia tuan-mudae* Becc. and *R. cantleyi* Solms-Laubach. Then histologically, the early pre-bud endophyte form within was observed in NIKOLOV & al. (2014) and MURSIDAWATI & al. (2019), showing the uniseriate strands and cell clusters within the host vascular cambium, respectively. In term of the whole process of the flower bud growth, *Rafflesia* requires at least 2-3 years in its meristematic stage inside the host plant of *Tetrastigma* [HIDAYATI & al. 2000]. The process of the flower bud development study by AMINI & al. (2019) in *R. cantleyi* revealed that *Rafflesia* has expressed different signaling transcription factors and genes involved with auxin, cytokinin, gibberellic acid (GA), abscisic acid (ABA), and jasmonic acid (JA). In a tissue culture study by SUKAMTO & MUJIONO (2010), the callus generation of the flower were successfully induced using a synthetic auxins, picloram and auxin-mimicking herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), while at the moment, a cytokinin zeatin was also added but shown no effect in callus growth and

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differentiation. As auxin regulates cell elongation and cytokinin regulates cellular division [TAIZ & al. 2015], how come auxin had more roles in SUKAMTO & MUJIONO (2010) callus generation study while cytokinin did not show any visible effect? Is there any auxin-related cellular differentiation (i.e. elongation) within the *Rafflesia* tissue during early development?

A previous study by MURSIDAWATI & SUNARYO (2012) focused on the flower bud development in more general approach. In the study, there are 3 phases of *Rafflesia patma* growth observed: 1) Phase I – host penetration during early germination stage, 2) Phase II – intrusion/invasive stage, where the flower bud starts to grow and affecting the host vascular tissue morphology, and 3) Phase III – flower establishment stage to matured flower bud prior to anthesis. Also this study, the perigone primordial, the processes primordial of the *R. patma* can be seen. Later updated in MURSIDAWATI & IRAWATI (2017), the fourth phase was added, Phase IV – conductive stage, where the flower bud is already established, taking nutrients from the host (host vascular tissue has been successfully hijacked), and preparing to the anthesis stage. However, this *R. patma* microscopical study in MURSIDAWATI & SUNARYO (2012) has actually more information and data, if the specimen is observed even closer. So using the unused data and available large resolution images from MURSIDAWATI & SUNARYO (2012) figure 3B, and 4A and B (used with permission), extensive new study is performed to analyze the *R. patma* cellular features from the condition of early flower bud differentiation until the later flower bud when the primordial bracts and perigone lobes are developed.

Materials and methods

Rafflesia patma samples

Rafflesia patma bud samples were taken from infected *Tetrastigma leucostaphylum* (Dennst.) Alston ex Mabb root section (to minimize the damage, compared to sampling the stem/aerial flower bud sample) on Pangandaran Natural Reserves, West Java, Indonesia at early and late stages of flower buds growth in 2012 [MURSIDAWATI & SUNARYO, 2012]. The early bud (Figure 2-4 in this study) was approximately 3 months, the later stage (Figure 5, a-c) was 4 months, 5 months, and 5 months (same age). The flower bud age was determined since as it swell on the host periderm. The endophyte – protocorm (primordial flower bud) transitional age inside the host cannot be determine since there was no visual clue to measure. The *R. patma* whole plant age also cannot be defined since, 1) the plant was from its natural habitat and during endophyte stage, the plant has no visual clue since it is microscopical (we couldn't even determine if the buds belong to the same *R. patma* individual) and 2) some *R. patma* within one host plants could be originated from primary infections (parasitic seed invades uninfected host) while the other might come with secondary infections (parasitic seed invades already infected host). Nevertheless, the timeline of *Rafflesia* individual growth stages can be seen in Figure 1.

Microscopical staining and image analysis

The microtomy results are from the unused microscope slides data (observed on Olympus CX31 optical microscope; Figures 2-4 in this study) and some images (Figure 5 in this study) are reproduced from MURSIDAWATI & SUNARYO (2012) with permission. The microscopical staining followed MA & al. (1993) with safranin staining, which colorized the tissue with tannin, lignin and suberin, and fastgreen, which colorized cytoplasm.

The microtomy procedure was using BERLYN & al. (1976) paraffin method and then sliced (microtomized) with Yamato RV-240 (Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and were sliced for 20 µm thick. This same procedure was later used for later study [MURSIDAWATI & al. 2019; MURSIDAWATI & al. 2020]. The pictures are enlarged and labeled during analysis in this observation using Adobe Photoshop CS6, while no labeling and extensive zooming were performed in MURSIDAWATI & SUNARYO (2012). The zooming in this study were performed to extensively analyze the *R. patma* flower bud cells and primordial tissue during differentiation as well as to observe the effect of the *R. patma* flower bud to the host (*T. leucostaphyllum*) tissue (mostly the vascular tissue: the secondary xylem, vascular cambium, and secondary phloem). The image background and scale bars were edited from MURSIDAWATI & SUNARYO (2012) also using Adobe Photoshop CS6.

Results

The overview of the early flower bud

At 3 month stage, where the flower bud tissue have not yet differentiated into flower organs, the flower bud tissue are composed of three types (based on the cell shape), separated within the proximal (closer to the host secondary xylem), middle, and distal region (closer to the host periderm), as can be seen in Figure 2. The first part is in the proximal region, where the flower bud tissue cells are elongated and proximal-distally oriented. The second part is the tissue with non-elongated cells in the middle. As the shape is more uniform and based on its location, the cell could developed into flower parenchyma tissue which shapes the flower, and in female flower, the middle of the flower is the place where the ovary of the flower developed [NAIS, 2001]. The third part is the tissue in the distal region. The tissue in this area is densely packed, the cell size is small, and highly meristematic. This meristematic cells located in the distal-most region to the small portion of lateral area in the middle region.

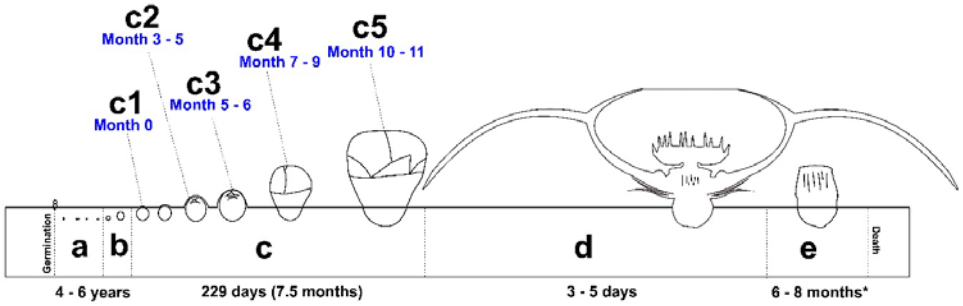

Figure 1. Timeline of a single *Rafflesia* from germination to endophyte stage (a), then to protocorm or primordial flower bud stage (b**)**, flower bud stage (c), anthesis stage (d), fruit stage (e), and death. During flower bud stage, there are sub-stages: Swollen host stage (c1), the flower bud age measurement starts after this stage. Then cupula stage (c2), where the flower bud swells in the host periderm layer, forming the cupula of flower. As the host periderm breaks, the flower bud enters the transitional cupula-bract stage (c3), before the bract fully emerges and becomes bract stage (c4). In the end of flower bud stage, the second transition occurs when the perigone lobes enlarge from the bract in bract-perigone stage (between c4 and c5), and finally the final stage of the flower bud prior anthesis, the perigone stage (c5). Of all age calculation, stage (a**)** and (b) cannot be determined since the stages are microscopic and has no visual clue. Ages in this figure is based on HIDAYATI & al. (2000), the naming of the flower bud stages is based on SUSATYA (2020). The flower bud ages are based on unpublished personal observation on *R. patma* in Bogor Botanical Garden, Indonesia.

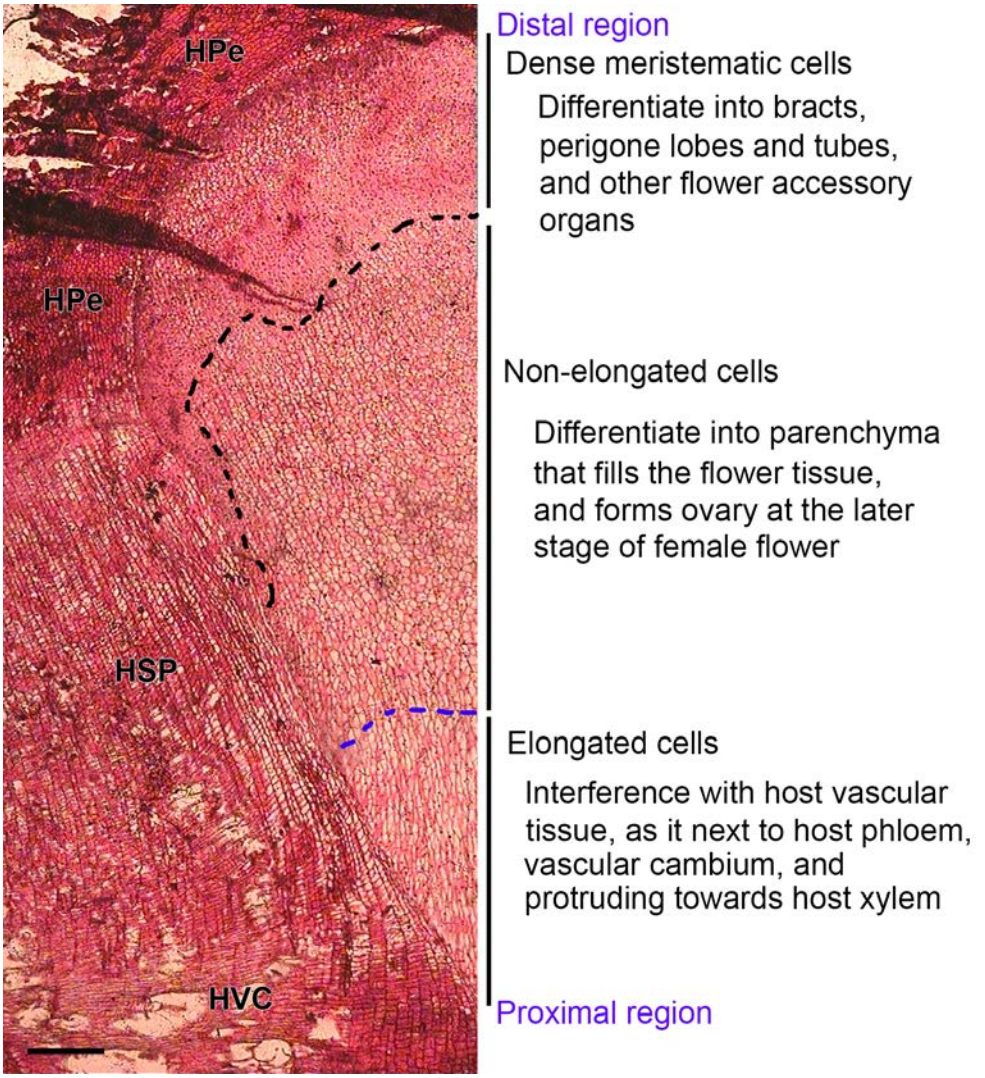

Figure 2. The three cell regions of the early *R. patma* flower bud saffranin-stained sample. At this stage, the cellular regions comprise of the dense meristematic cells at the distal region where it can differentiate into protective bracts, perigone lobes, and central disc (see later at Figure 5), the non-elongated cells at the middle region where the cells grows into parenchyma cells and where ovary supposed to develop at female flower, and the elongated cells at the proximal region where the tissue is pointing towards the host xylem area. HPe – Host periderm, HSP – Host secondary phloem, HVC – Host vascular cambium. Scale bar = 1 mm. The black and blue dots separate the cell regions. The dark area at the distal region is the bent HPe tissue during microtomy slicing. Note: This same image in this figure is enlarged in Figure 3 and 4. The picture are compiled from 4×10 magnification microscopical images. Scale bar = 1000 µm.

The distal region of the *R. patma* early flower bud

The distal region are packed with meristematic tissue. When observed closer in 10×10 magnification, the distal-most region is the where most meristematic cells proliferates, signified by the smaller-sized cells with greater quantity than its surroundings (Figure 3; *R. patma* meristematic distal-most region – RMD). Proximally from the distal region, some cells appears uniform in sizes and appears to have completed their cell divisions, but some are still also appear meristematic (Figure 3; *R. patma* meristematic region – RM).

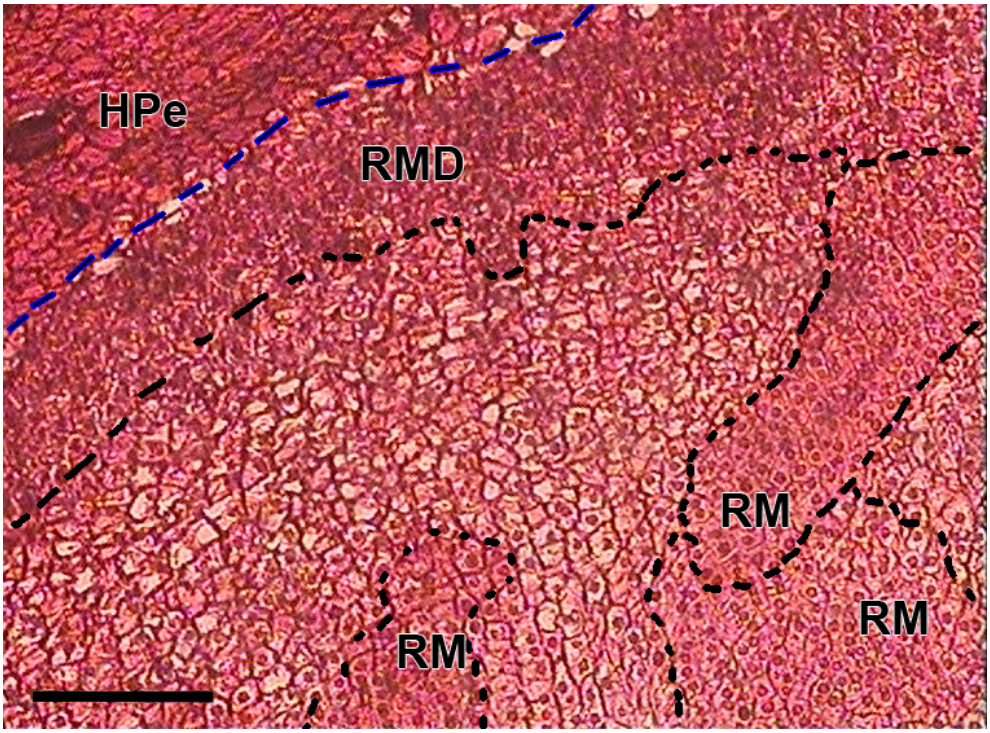

Figure 3. The heavily and dense meristematic part of the *R. patma* flower bud distal (apical) region**.** This area supposedly grows the flower bud further to the host periderm (HPe) area, as the *R. patma* meristematic distal tissue (RMD) (close to intersection with *T. leucostaphylum* tissue; black-blue dotted intersection) proliferates, The *R. patma* meristematic tissue (RM) shown with densely packed cells bordered with the dotted regions. This are could be where the protective bracts and perigone lobes developed. Note: This figure is the same sample and enlarged from Figure 2. Magnification 10×10. Scale bar = 250 µm.

The basal/proximal region of the *R. patma* early flower bud

In the middle region of the flower bud, the cell sizes are larger than in the distal region and the sizes are relatively uniform (Figure 4, a and b; see *R. patma* parenchyma tissue – RP) and supposedly matured as parenchyma tissue. In the lateral size, the dense, smaller meristematic cells can also be found where it extended from the distal region (Figure 4b; see *R. patma* meristematic lateral tissue – RML). The flower bud tissue in this study are surrounded by the host (*T. leucostaphylum*) secondary phloem region (Figure 4 and 5; see host secondary phloem – HSP). However, until now the method of *Rafflesia* to obtain nutrient is still unknown.

As the mature *Rafflesia* flower relies on xylem and vascular parenchyma to distribute nutrients [MURSIDAWATI & al. 2020], it is possible that the outermost parenchyma cells of *Rafflesia*, in this case *R. patma* contribute in nutrient gathering from the host phloem close to it. The parenchyma direction at the host-parasitic intersection border of this region is that the parenchyma cells are oriented in parallel to the laterally surrounding phloem.

In the proximal region of the flower bud, the tissue cells are elongated (Figure 4; see *R. patma* elongated cells – RE), with proximal-distal cell orientation. Compared to the parenchyma cells in the middle region, is at the host-parasitic intersection border, the elongated parenchyma cells (we assumed that those are still parenchyma cells despite in different shape) in this region is oriented almost perpendicular to the laterally surrounding host secondary phloem. On the later stage (Figure 5c), these elongated parenchyma cells are fully perpendicular oriented towards the host secondary xylem at the proximal end host-parasitic border.

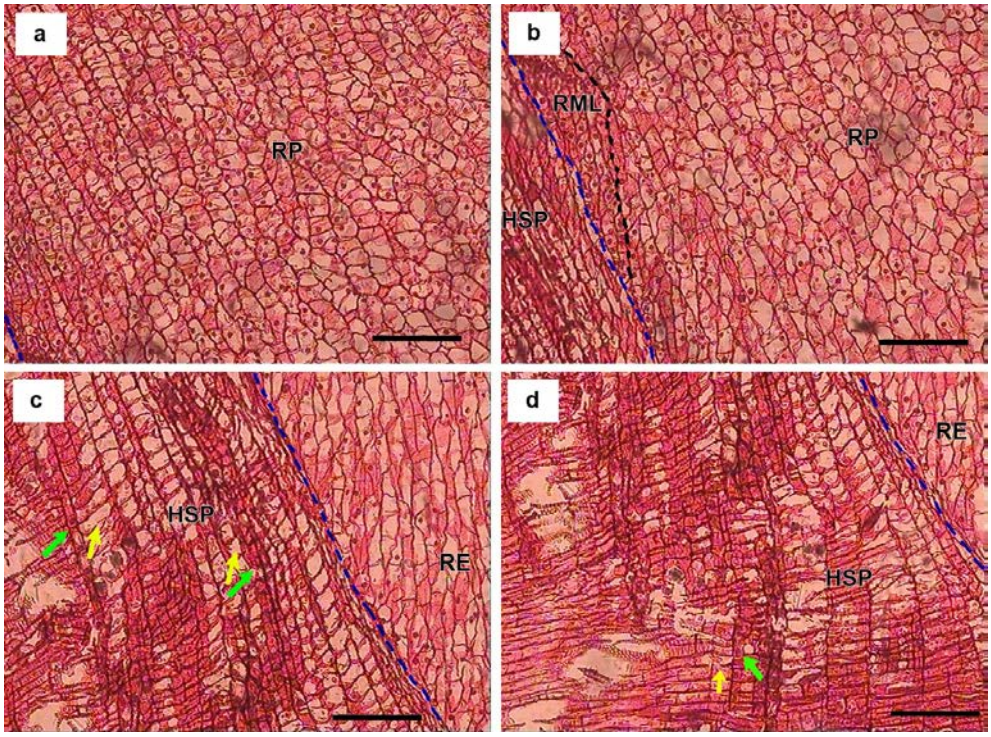

Figure 4. The middle (a and b) and proximal region (c and d) of *R. patma* side-by-side with *T. leucostaphylum* tissue layer. The observed *R. patma* tissue are mostly the non-elongated parenchyma cells (a) at the middle area of the bud tissue. At the intersection area (b) the tissue has the lateral meristematic cells (RML) signified with the smaller size of the cells extended to the distal region, and located close to the host-parasite border (blue dots) next to the *T. leucostaphylum*, and the RP of *R. patma*. Closer to the proximal region (c and d), there are no transitional meristematic cells seen, but the *R. patma* cells are more elongated (RE) and located directly to the host-tissue intersection borders (blue dots) next to the *T. leucostaphylum* (host) secondary phloem (HSP) layer. This HSP layer can be identified by the phloem companion cell (green arrows) and the sieve tube cells (yellow arrows). Note: This figure is the same sample and enlarged from Figure 2. Magnification 10×10. Scale bars = 250 µm.

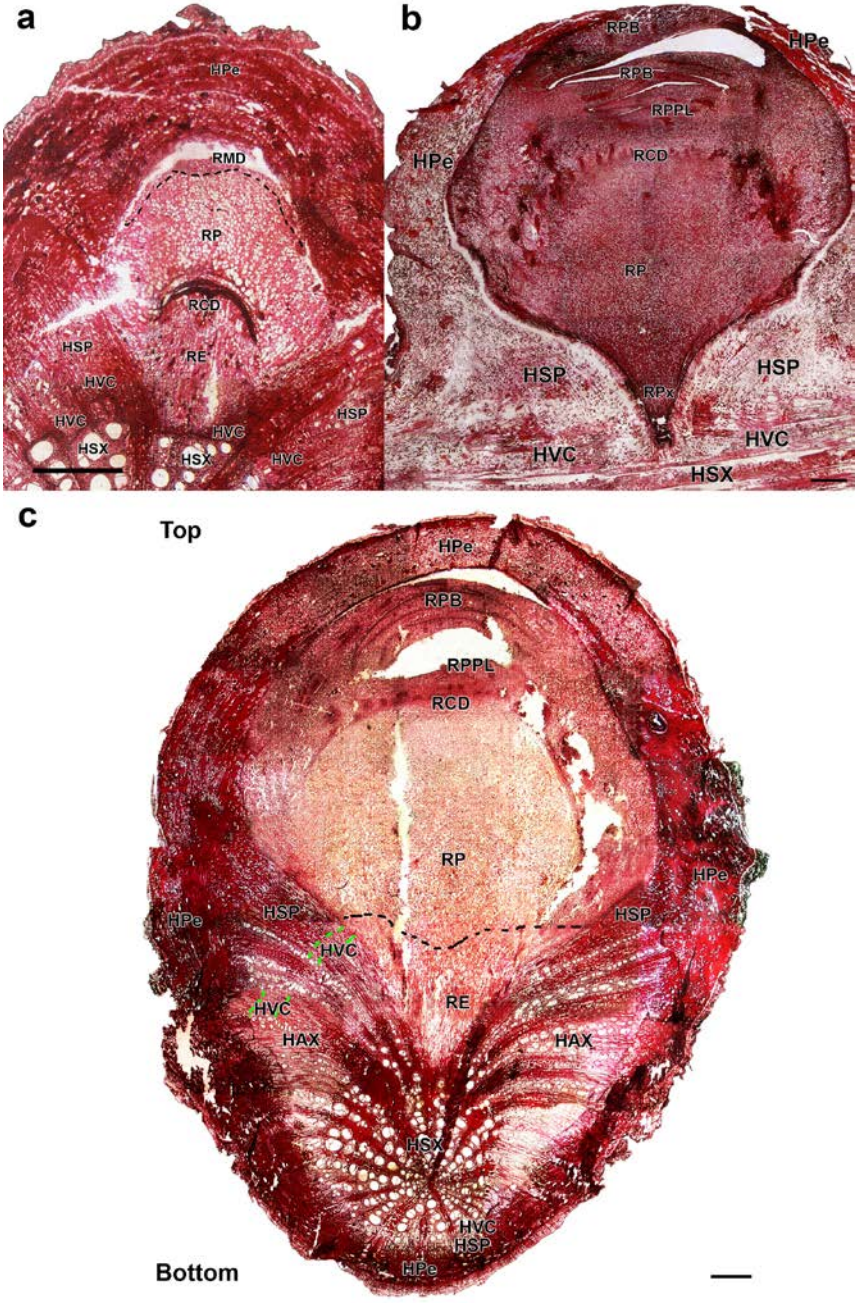

Figure 5. Transition of early to late stage of *R. patma* flower bud development and its interaction with the host, *T. leucostaphyllum* tissue. At early stage (a), the primordial central disc of *R. patma* area (RCD) can be seen distally to the elongated cells (RE) and proximal to the *R. patma* parenchyma cells (RP). At the most distal area of this stage, there are still no bracts or perigone lobes primordial, yet the

distal meristematic area (RMD) with small-sized dense tissue can be seen. The flower bud is still covered by thick layer of the host periderm layer (HPe). In the proximal region, the *R. patma* flower interrupts the growth of host vascular cambium (HVC), making no phloem growth at the bud area. Later on the later stage (b), for longitudinal cross section and **c** for transversal cross section, the flower bud elongated cell area (RE) has interfere many part of the host tissue growth. Especially can be seen in **c**, the vascular cambium (HVC; marked with green dots) area at the top are pushed laterally by the *R. patma* parasitic tissue, and thus are affecting the xylem growth and resulting at the host anomalous xylem area (HAX), as for host secondary phloem (HSP) compared to the bottom part of the *T. leucostaphyllum* where the HVC and HSP are still able to be found as orderly concentric vasculature. In this stage, *R. patma* has more RP, engorging it towards HPe. The RMD has developed RCD, primordial perigone lobes (RPPL), and primordial bracts (RPB). It will continue to grow toward HPe (from c to b), where the bud tissue breaks HPe, revealing the RPB part of *R. patma*. Some cracks can be seen in (a) and (c) due to the fragility of the tissue during microtomy slicing. Pictures are compiled from 4×10 magnification microscopical images. Pictures are reproduced and edited by permission from MURSIDAWATI & SUNARYO (2012). Scale bars = 724.4 µm.

The late development of flower bud

At the late flower bud, it appears that the primordial central disc are formed first (Figure 5a; the bud age: 4 months), and in the next stage, the primordial structures of early processus are formed (Figure 5b and c; the bud age: 5 months; see RCB – *R. patma* primordial central disc). Then, the primordial of protective layers, the perigone lobes and bracts are formed (Figure 5a to c and to b; where the *R. patma* distal meristematic region – RMD in Figure 5a, developed into *R. patma* primordial perigone lobes – RPPL, and *R. patma* primordial bracts – RPB in Figure 5c; and finally in Figure 5b, the bract becomes exposed as the host periderm – HPe breaks; also known in cupule-bract transition stage in SUSATYA (2020) where the emerging bract color is still pale). Despite the bud ages are the same, bud in Figure 5b could emerge first probably determined by how thick is the peridermal layer (the root cork) of the host at the bud growth area. Also at the later stage, the proximal region of the flower bud appears to forms a pointed tissue towards the host secondary xylem region (Figure 5b and c). It shows that instead of occupying the entire region of xylem as sinker cells (observed in *Cytinus*; DE VEGA & al. 2007; and *Pilostyles*; KUIJT & al. 1984), *R. patma* proximal region "sinks" only towards 1 or 2 vascular bundles as shown in Figure 5a (approximately it attached to 2 vascular bundles area) and 4c (approximately attached to 1 vascular bundle area). However, despite the low coverage of the host vascular bundle, the flower bud proximal growth greatly alters the growth and positioning of the host vascular cambium and host secondary phloem (Figure 5a and c). On the other hand, the flower bud lateral growth also presses the host vascular cambium laterally, to the point that the host vascular cambium anomalously form abnormal xylem growth (Figure 5c; see host anomalous xylem vasculature – HAX) and the host secondary phloem in the affected area bent and grows laterally. In Figure 5c, it can also be seen the great difference between the *R. patma* affected *T. leucostaphylum* tissue (top area) and the unaffected *T. leucostaphylum* tissue where the vascular bundles are uninterrupted and growth concentrically as normal (from proximal to distal: secondary xylem, vascular cambium, secondary phloem, and periderm).

Discussion

The composing cell differences in early flower bud different regions

Rafflesia patma cellular division rate seems to be differ on each stage. During endophyte stage, the *Rafflesia* cells can be distinguished from its surrounding host cells by its large nuclei appearance (also seen in this study, where *R. patma* cells are more visible than its surrounding host cells), which might lead to possession of large genome size, leading to slow growth due to longer time required for genome replication [NIKOLOV & al. 2013]. At this later stage (this study), it appears that the cell division of *R. patma* cells happen more rapidly. It is not yet clear if there a change in molecular level which regulates this shift of cellular proliferation rates.

The distal region of the early stage of the flower bud (age: 3 month) are composed mainly by the meristematic cells. This part serves as the "apical part" in analogue to the other plant where the apical meristem. The middle region is composed of the non-elongated parenchyma cells, while in the proximal region is composed of the elongated parenchyma cells. Based in the study in MURSIDAWATI & al. (2019), *R. patma* grows from the host vascular cambium, forming the "basal part" in the proximal region of the *T. leucostaphylum* tissue where the host secondary xylem can be found, then the rest of growth are occurred distally towards the host secondary phloem layer, and finally to the periderm. At early stage (endophytic-protocorm transitional stage, as in MURSIDAWATI & al. 2019), the proximal cells of *R. patma* is still not as elongated as in the early flower bud growth in this study. However, the distal region is already composed by the meristematic, proliferating cells.

Apparently, parenchyma tissue in the early stage between the middle and the proximal region has difference in orientation towards the laterally surrounding host-parasitic border. It is unclear if there are differences of functions between the parenchyma tissue in the middle and in the proximal region. The host-parasitic border parallel parenchyma tissue in the middle are laterally surrounded by host secondary phloem. It is possible, that the parenchyma cells could help to acquire the nutrients from the host tissue. The case in *Cytinus*, despite the parasitic tissue possess its own parasitic xylem and phloem, no xylem-xylem and phloem-phloem [DE VEGA & al. 2007], thus it is proposed that the parenchyma tissue is helping the nutrient distribution by cellular wall between the host and parasite via apoplastic flow continuum [COETZEE & FINERAN, 1987; KUO & al. 1989]. DE VEGA & al. (2007) also mentioned that the parenchyma cells with thickened walls may act as transfer cells, providing interaction between host and parasite, helping with nutrient absorption, transport, and distribution of the photoassimilates between the host and parasite. In the *Cytinus* study by DE VEGA & al. (2007), no parenchymal cell shape difference and orientation detected, as it just serves as the endophytic tissue surrounding the parasitic xylem and phloem, adjacent to either host phloem or xylem. It is unclear if the cell shape and orientation have specific application to the *Rafflesia* bud. Reflecting to this condition, it is possible that the proximal region parenchyma of early flower bud helps with photoassimilates absorption, transport, and distribution from the adjacent host phloem via the apoplastic flow mechanism as in *Cytinus*, or it points proximally as it specifically absorbs the photoassimilates from the host phloem. Additionally, as the elongated parenchyma cells are oriented proximal-distally and seemingly pointed toward the xylem, Figure 5b and c show that the contact are with xylem is not actually visible (no visible intrusion or side-by-side intervention) compared to the direct phloem contact which is just laterally in the bud proximal region. The proximal point of late flower bud is rather conical in shape, suggesting that it could be analogous to the root function and if the absorption occur only on the elongated cell of the bud, its function is possibly be to regulate as well to optimize the nutrient absorption despite the abundance of the host secondary

phloem in the middle-proximal region of the bud and avoid too much absorption when the flower bud grows bigger which could affect the host condition. Up to date, it is known that *Rafflesia* does not stores starch on its tissue, compared to the surrounding host tissues (NIKOLOV & al. 2014; observed at the same late flower bud stage in WICAKSONO & MURSIDAWATI, 2020), indicating that *Rafflesia* directly takes the sugar photoassimilates from its host and this requires intake regulations.

This suggestion, however, have not been documented or studied and it is still possible if the non-elongated parenchyma also takes role in nutrient absorption as in some certain condition (Figure 5c) the non-elongated parenchyma position is closer to the host phloem rather than the elongated ones which are closer to the host xylem vascular bundles. This penetration towards between the host xylem vascular bundle regions might provide better grip as the bud grows into fully developed flower, hence making it more analogous to the taproot. Additionally, despite there are no sign of xylem intrusion, the bud elongated parenchyma cells closer to the host xylem could serves as water collector. Also this elongated parenchyma cell morphology is also similar to the vascular parenchyma cells as in MURSIDAWATI & al. (2020), further suggesting its function in nutrient distributions from host.

In term of differentiation of the elongated parenchyma cells, it is unclear if auxin plays some role in the cellular elongation from early condition of parenchyma cells shown in MURSIDAWATI & al. (2019). Cell elongation by auxin is mediated by loosening the cell wall, allowing more water into the cell [MAJDA & ROBERT, 2018]. As the most proximal region elongated cells are also located in proximity to the host xylem as the water source, this positioning might also explains how the parenchyma cells get elongated possibly by auxin regulation. Auxin has been studied to initiate the flower primordial [ALABADI & al. 2009; FAN & al. 2015], but the cytokinin affects flower bud formation, size, ovule formation, and seed size [VAN DER KRIEKEN & al. 1989; BARTRINA & al. 2011]. However, it is unknown if the auxin is synthesized on the distal region of *Rafflesia* (analogue to the apical shoot; TAIZ & al. 2015), or host-originated. The elongation of the cells also require further physiological studies. As the *in vitro* study of *Rafflesia* is extremely hard to perform (so far, only SUKAMTO & MUJIONO (2010), has succeeded in callus generation but not followed by somatic embryo or organ generation), new procedure is required to observe the effect of added auxin and cytokinin to the flower bud of *Rafflesia*.

Differentiation of processes (central disc), perigone lobes, and bracts

The flower accessory organs of *R. patma* differentiation in this study appears to be in the later stage of flower bud (after 4 month old). In Figure 5a, the first to differentiate is the central disc, which later (Figure 5b and c), forms the spiky processes. At the same time (Figure 5c, then b), the primordial perigone lobes developed close to the central disc. Within this region of development, the perigone tube and its diaphragm will grow in much later stage. Compared to the perigone lobes, the bract developed first. This is because the bract serves as the protective layer to the flower once it emerged. According to SUSATYA (2020), the flower bud of *Rafflesia* starts as in cupula/cupule stage, then bract-cupule transition where the bract is emerged as a pale early bract, which later hardened and darkened in the next stage, bract stage where the bract is fully emerged and covering the flower bud distal region. Then the bract-perigone (lobe) transition stage where the bract revealed the perigone lobe within partially, perigone stage where the perigone lobe fully enlarged and revealed, and finally the *Rafflesia* blooms/anthesis stage. The fully emerged and hardened bract provides protective shell to the flower. Later, the protective early perigone lobe with smooth, waxy abaxial will open first before the late perigone lobe with rough, leathery abaxial [MURSIDAWATI & al. 2020].

The affected host vascular growth at later stage of flower bud

As previous studies [NIKOLOV & al. 2014; MURSIDAWATI & al. 2019], *Rafflesia* tend to grows in the *Tetrastigma* with secondary growth. Using the reference of the grapevine (*Vitis* sp.; also Vitaceae as *Tetrastigma*) root growth, which has the same anatomy, as the root grows from its primary growth stage to the secondary growth stage, the root cortex along with the endodermis will be pressed distally, forming the cork or periderm, the pericycle fills the portion where the cortex was as it moves distally as well, and contributes to the vascular cambium development (reference from hybrid grapevine, *Vitis berlandieri* × *rupestris* in GAMBETTA & al. 2013). It was known in the dicot secondary growth of root, the pericycle plays important role in periderm and vascular cambium development, aside to its role to develop lateral roots [BECK, 2010]. This anatomical details are important to determine the anatomical location of the *Rafflesia* flower bud.

During the later stage of flower bud (Figure 5, from a to c, to b), it appears that the distal growth will dominate, resulting to the smaller bud tissue in the proximal region and larger in the distal region. The impact of flower bud extensive growth in the late stage can be seen in both Figure 5a and c. The *R. patma* flower bud, as it grows from the vascular cambium towards the host periderm [MURSIDAWATI & al. 2019] will press the surrounding *T. leucostaphylum* vascular cambium layer and secondary phloem. As *T. leucostaphylum* vascular cambium layer pressed laterally, the organogenesis of the new vascular tissue will be altered. The new xylem located proximally from the flower bud will be no longer generated from the vascular tissue and similarly, no phloem will be generated from the pressed vascular tissue. Leaving only xylem in the *Rafflesia*-inhibited vascular bundle. The surrounding vascular bundle will be pressed laterally, causing irregularity in the vascular cambium phloem and xylem generation (Figure 5c; top area). Interestingly, host secondary phloem will be generated even longer surrounding the late flower bud proximal area, hence assumingly provide the flower with more nutrients. This alteration of host can be also observed in the other parasitic plant infestation of *Phoradendron crassifolium* (Pohl. ex DC.) Eichler on *Tapirira guianensis* Aubl., where its xylem lumen size are decreased, higher density of embolized vessel, higher vessel density, taller and wider rays, fibers with thinner cell wall, and the xylem growth bent laterally due to the parasitic growth [TEIXEIRA-COSTA & CECCANTINI, 2015]. The difference here is the xylem is mostly affected, especially *P. crassifolium* is a hemiparasitic plant.

The alteration of *T. leucostaphylum* root tissue by the *R. patma* bud tissue during late development occurred in 1-2 vascular bundles area only, leaving their vascular growth completely inhibited, few adjacent vascular bundle to grow with anomalous xylem area and elongated phloem area (Figure 5c, top). The rest of the root however, are continued to grow normally (Figure 5c, bottom). This strategy of minimizing host vascular damage probably is to allow the host to be alive as *Rafflesia* flower is massive (hence required large amount of nutrients), with chances of other flowers bud is growing in the same host plant (Figure 6). This 1-2 vascular inhibition (compared to *Phoradendron* where it affects multiple vascular bundles) reduced the chance of the host *T. leucostaphylum* death, which may lead to the death of the entire *Rafflesia* endophytes inside the host. In *Rafflesia* (referring to Figure 6), the scale of host vasculature damages are increased if multiple buds are growing in the different directions. However, if multiple buds are growing in the same direction (in series), the vasculature damages are assumed to be minimal. This condition would apply as a comparison of multiple buds grown in the same host root size. If the host root is larger, with more vascular bundles, the damage could be even more minimalized.

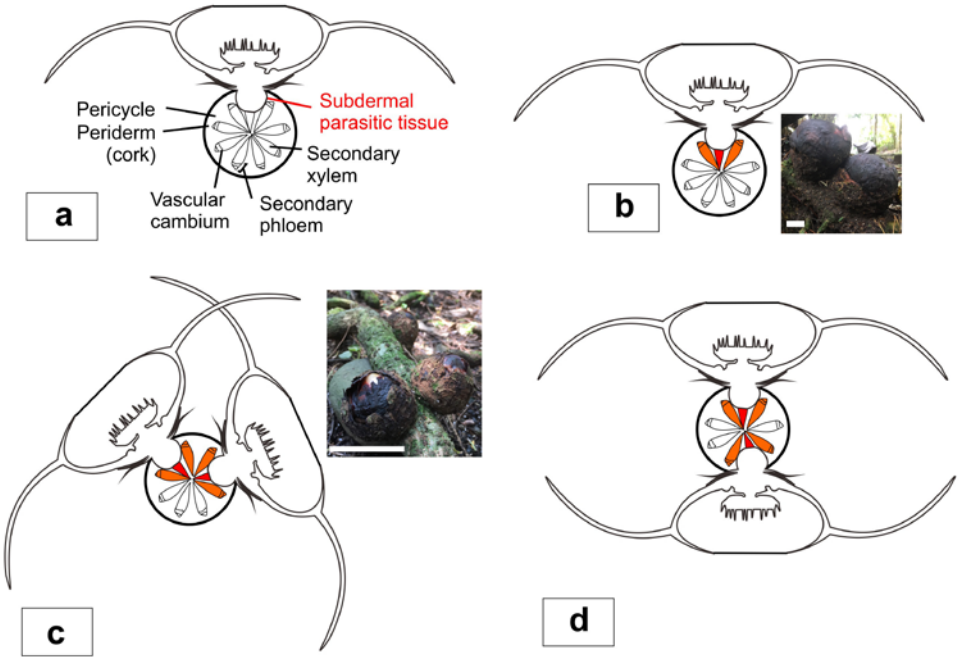

Figure 6. Interaction of *Rafflesia* and its host root in general. The host, *Tetrastigma* organ where *Rafflesia* grows is the root which has developed under secondary growth (it has cork layer, vascular cambium, the pericycle fills the area where cork previously is, and the epidermis and endodermis are no longer exist) (a). In this study, a single *Rafflesia* flower grows in a single vascular bundle cambium region (b), suppressing the secondary phloem and xylem growth in this one respective bundle (red), and affecting the neighboring bundles growth pushing laterally to make the bundles to become adjacent to the parasitic tissue (orange). It is suggested that their secondary phloem tissues become nutrient providers to the parasitic tissue. In case of multiple buds growth within the same vasculature line (in series and the same direction), the flower can also grow (inset; photo taken from September 2017). Other case in multiple buds growth, if both buds growth in some angle of angle difference (c) (and the inset; photo taken from July 2018), that means more damage to the host and more vascular bundles are affected. However, as *Rafflesia* affects the host vasculatures in at least 3 bundles, same host vascular damages as in (c) if both flowers are in opposite directions despite different scale of possible host tissue truncations (d). The host damage will be increased if more buds are growing in multiple direction angles, less damage is assumed if there are more buds bud in the same growth direction. The host stem size in a, b, and c are assumed at the same size. If the host size is larger, the host vasculature damage could be different. Photos are taken by Adhityo Wicaksono and are unused data. Scale bars = 5 cm.

Conclusion

The flower bud of *R. patma* has differences in the early stage and the late stage of development. On the early flower bud, the bud has three types of cells: densely packed and meristematic distal region, non-elongated parenchyma in the middle region, and elongated parenchyma in the proximal region. The distal region is contributing to flower first protective layers (bract and perigone lobes) development as well as the flower accessory organs (central disc with the processes). The non-elongated parenchyma in the middle region fills in the

flower tissue, helps in possible nutrient transport and distribution from the surrounding host secondary phloem. However, the elongated parenchyma might be more specifically to absorb nutrients from the host and provides structural grip, making it analogous to the root. The late flower bud develops primordial central disc first, followed by the primordial bract and perigone lobes. Also on the later stage of flower bud development, the flower bud enlarged and inhibits the growth of 1-2 host vascular bundle, leaving only the early xylem. Surrounding it, the host vascular growth are altered heavily with enlarged secondary phloem region and anomalous xylem region developed. On the other side of the host however, no disturbance is occurred and the root vascular tissues grow normally. This strategy of minimalizing vascular damage is assumed to prevent host death if multiple *Rafflesia* bud is emerging at the same time in the same direction.

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Adhityo WICAKSONO – is a researcher and head of biotechnology division of Genbinesia Foundation, Indonesia, and a PhD student in Åbo Akademi University, Finland. He works in multidisciplinary perspective of biology. For the past 5 years, he has been working and focusing on the research of the biology of Rafflesiaceae and plant biotechnology.

Conflict of interests

All authors declare no conflict of interests.

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MICROPROPAGATION OF *MUNTINGIA CALABURA* L. AND ASSESSMENT OF GENETIC FIDELITY OF *IN VITRO* RAISED PLANTS USING ISSR AND RAPD ANALYSIS

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Abstract: *Muntingia calabura* L. is a potent medicinal plant. Because of that several researchers reported phytochemical and pharmacological studies, but only minimal information is obtainable on tissue culture and genetic stability evaluation studies. Therefore, in the present investigation we attempted to establish a reliable direct and indirect regeneration study through leaf and node explants on MS medium containing cytokinins BAP and KN (0.5-3.0 mg l^{-1}) in combination with auxins IAA and NAA (0.5 mg l^{-1}). The breakage of bud and shoot initiation were noticed with the initiation of callus at lower concentrations of auxin alone at 0.5 mg l^{-1}. 2,4-D was found to be good in callus induction from leaf and nodal explants. A problem of browning callus was prevented by regular subculturing of callus cultures. Leaf explants exhibited maximum number of shoots (32±0.88a) with shoot length (7.6±0.17a) and nodal explants obtained optimal number of shoots (26±0.88a) with shoot length (9.6±0.30a) on MS medium supplemented with BAP (2 mg l^{-1}) and IAA (0.5 mg l^{-1}). Half strength MS medium fortified with IBA (2.0 mg l^{-1}) was effective and achieved 70% of rooting. These well-developed plantlets were shifted to pots containing soil and vermicompost in 1:1 ratio for acclimatization. The acclimatized plants were field transferred with survival rate of 85%. The ISSR and RAPD markers analysis revealed the genetic stability of *in vitro* regenerated plants with the mother plant.

Keywords: genetic fidelity, ISSR, *Muntingia calabura*, RAPD, regeneration.

Introduction

Muntingia calabura L. is a rapidly growing tree, domestic to the American continent, and is commonly cultivated in warm areas of the Asian region. The *Muntingia calabura* is also known locally as "Jamaican cherry", belongs to the Family Muntingiaceae. *Muntingia calabura* is regularly grown as roadside trees [MORTON, 1987; YUSOF & al. 2011; ZAKARIA & al. 2006a,b, 2007a-f, 2008, 2010, 2011]. The different plant parts of *Muntingia calabura* possess several potential medicinal uses in herbal medicine for the treatment as antiseptic [ZAKARIA & al. 2006a], antipyretic [ZAKARIA & al. 2007f], antiulcer [IBRAHIM & al. 2012], antidiabetic [SRIDHAR & al. 2011], antihypertensive [SHIH & al. 2006], cardioprotective [NIVETHETHA & al. 2009], anti-inflammatory, antinociceptive [ZAKARIA & al. 2006a], antibacterial [YASUNAKA & al. 2005] and antiplatelet aggregation [CHEN & al. 2007], especially in leaves and roots. This plant is rich in flavonoids, flavones, and flavanones, rendering to its potent antitumor activities [KANEDA & al. 1991]. Despite of its traditional claims, different parts of the plant have been used to treat various ailments.

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Muntingia calabura root extracts are screened for cytotoxic activities against A549 and HT-29 cells [KANEDA & al. 1991]. Plant parts like leaves, roots, and stems were yielded cytotoxic flavonoids: Chrysin 2, 4-Dihydroxychalcone, and galanin 3, 7-dimethyl ether. The boiled bark is used to reduce the swelling in the lower extremities [ZAKARIA & al. 2006a]. Whereas, the leaves decoction or steeped water is employed to decrease ulcers in the stomach, prostate gland swelling and to alleviate headache and cold [MORTON, 1987]. The fruits are often cooked and made into jams. The Flowers are used as tranquilizers and tonic in Colombia [PEREZ-ARBEALAEZ, 1975].

There is a need to proliferate (or) multiply wider uses by conventional and biotechnological approaches. The improvement of the tree by traditional breeding is a delayed process due to its long juvenile period and high heterozygosity [SINGH & al. 2002]. Micropropagation of tree species offers a renewable biomass, conserve the genetic traits and produce clonal saplings for afforestation [ROUT & al. 2008]. Several plant species, especially woody plants are recalcitrant to high frequency root and shoot regeneration due to their *in vitro* recalcitrance [MCCOWN, 2000]. *In vitro* regeneration studies are difficult in woody tree species due to the high presence of phenols and alkaloids, which leads to the problem of browning callus. At the same time as, many of the researchers have established protocols in *Gardenia latifolia* Aiton, *Elaeocarpus serratus* L., etc. The present investigation was aimed for the development of sustainable regeneration protocol for *Muntingia calabura* and the influence of various plant growth regulators (PGRs) in the micropropagation. The regenerated plants were analyzed for genetic homogeneity studies using ISSR and RAPD markers. Moreover, the *Muntingia calabura* is growing at pollution releasing areas such as roadside and also heavy metal-containing areas. In future *in vitro* regenerated acclimatized plantlets can be used for isolating and screening of secondary metabolites from various plant parts and also these acclimatized plantlets roots are treated with bioinoculates (PGPR, *Azotobacter*, PSM and *Mycorrhiza*) for evaluating their survival rate in abiotic stress conditions.

Material and methods

Explants source and surface sterilization. The fruits of *Muntingia calabura* were squeezed and mixed in soil, later they were rubbed in sandy soil to separate the seeds. The seeds were used for the establishment of healthy plants in the polyhouse and field of Department of Biotechnology, Kakatiya University, Warangal, Telangana State, India. Young leaves, nodes, internodes, stem and other explants of *Muntingia calabura* were collected from the polyhouse or field during the month of January to December. The shoots were excised into 1.5 cm nodal segments and leaves were cut into 1 cm rectangular bits. Then the explants were washed rigorously under running tap water for 15-20 minutes for removing dust particles on the surface of explants. Then followed by surface sterilization by 1% Bavistin (a fungicide) solution for about 5 minutes and cleaned 3-4 times with sterilized double-distilled water. The explants are now transferred into laminar airflow for further sterilization. The explants (leaf and node segments) are now sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for about 2-3 minutes and then rinsed again with sterilized distilled water to remove any traces of $HgCl_2$.

Culture medium and conditions. MS medium [MURASHIGE & SKOOG, 1962] was prepared by adding 30 g/l sucrose, 100 mg/l Myo-inositol and with varying concentrations of cytokinins BAP or kinetin KN (0.5-3 mg l^{-1}) and auxins IAA or NAA (0.5 mg l^{-1}). The pH of

the medium is set to 5.6±0.1 with 1N HCl or 1N NaOH before the addition of agar (0.8%). Now the culture media was sterilized in an autoclave with 1.1 kg cm^{-2} pressure at 121 °C for 15 min. The surface sterilized explants were inoculated onto the sterilized culture medium (one explant per culture tube). All inoculated cultures were transferred into a controlled condition chamber for the incubation at 25±2 °C temperature with a relative humidity of 65±5% under a 2000-2500 lux for 16h Photoperiod. Data was collected and analyzed after a period of 45 days.

Callus induction and shoot proliferation. Initially after two weeks, brown and light green color callus was initiated from cut margins of leaf and node explants cultured on MS medium containing 0.5 mg l^{-1} 2,4-D. After the third week, the brown and thick green callus expunged was subcultured onto medium with 0.5 mg l^{-1} IAA or NAA with various concentrations of KN (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg l^{-1}) or with BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg l^{-1}) to identify the utmost appropriate combination of PGR's for the multiplication of regenerated shoots from leaf and node segment of *Muntingia calabura.* The data on percentage of regeneration, number of regenerated shoots per explants and their shoot length (cm) was recorded after fourth week of culture.

Rooting of in vitro regenerated shoots. After achieving healthy and elongated *in vitro* regenerated shoots upto a height of 1.0 to 1.5 cm, they are excised and cultured onto 0.8% agar-gelled with half strength MS medium fortified with various concentrations of IBA 0.5, 1.0, 1.5, 2.0, 2.5 mg l^{-1} for induction of *in vitro* rooting from regenerated shoots. The percentage of rooting, number of roots and their length (cm) was recorded after fourth and fifth week of culture.

Hardening and acclimatization. The *in vitro* regenerated plantlets were carefully removed from the culture vessels and washed thoroughly with sterile distilled water to get rid of adhered agar and then transplanted into plastic cups filled with sterile garden soil and vermicompost (1:1) and maintained inside a plant growth chamber at 25±1°C under 16hr daylight provided by white fluorescent lamps. After acclimatization, these plantlets were transferred under greenhouse conditions and covered with polythene sheets for maintaining moisture and humidity for further acclimatization. After sixth and seventh weeks these acclimatized plants were hardened by transplanting into large pots filled with garden soil and manure. The percentage of survival was recorded after 3 weeks of the field transfer and found to be 85% same to the mother plant.

Genomic DNA Isolation and genetic homogeneity analysis. The genomic DNA was isolated from the mother plant and regenerated plantlets by using cetyltrimethyl ammonium bromide (CTAB) method [DOYLE & DOYLE, 1990]. The DNA concentration was checked by 0.8% agarose gel electrophoresis. Assessment of genetic fidelity of *Muntingia calabura* plants was performed through Inter-Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) analysis. Each set of 10 ISSR and RAPD primers was selected for the genetic stability study. The PCR reaction mixtures and conditions are followed based on the protocol described by PENDLI & al. (2019). The PCR bands were calculated using the 100 bp DNA ladder (Himedia). The experiments were repeated thrice to verify the reproducibility of PCR bands.

Data analysis and observations. The cultures were regularly subcultured onto fresh medium with an interval of 4-5 weeks. The cultures were observed and recorded after every six days of inoculation. All the experimental work was conducted thrice with 60 explants for each combination. The significant differences for various parameters recorded were calculated by mean and standard error variance (ANOVA) using SPSS software.

Results and discussions

The sterilization treatments of explants with 0.1% (w/v) mercuric chloride ($HgCl_2$) yielded 95% of contamination-free leaf and nodes of *Muntingia calabura*. The responses of both the explants inoculated shown the capacity of shoot regeneration differently to the two cytokinins (BAP and KN) and combination with (IAA and NAA) with various concentrations of PGR's. The 15-day callus subcultured on MS modified medium showed efficient shoot induction. The age of explants showed to play a key role in morphogenesis [DISTABANJONG & GENEVE, 1997].

Callus induction from leaf and node explants. *Muntingia calabura* is a woody tree species and contain a wide variety of secondary metabolites like alkaloids or phenols, the browning of the medium may be due to the production of alkaloids or phenols in the medium. The mixture of antioxidant was found to reduce the phenolic exudation and browning of callus in *Ceratonia siliqua*, *Pterocarpus santalinus* [ROMANO & al. 2002; PRAKASH & al. 2006]. Callus was induced from the excised portions of midrib and lateral veins of leaf lamina and basal part of nodal regions of the node. The initiation of callus was achieved even at lower concentrations of auxin alone with 0.5 mg l^{-1} 2,4-D. 2,4-D found to be good in inducing callus from leaf and nodal explants (Figure 1a and Figure 2a). 2,4-D plays a major role in callus induction in an ample range [RANI & GROVER, 1999]. An identical report on high percentage of callus induction was obtained from stem and leaf explants on MS medium with IAA (0.5 mg l^{-1}) and 2,4-D (1.0 mg l^{-1}) in *Citrullus colocynthis* [SHASTHREE & al. 2012] and *Momordica cymbalaria* [CHAITANYA & al. 2020]. A problem of browning callus in the node segment (Figure 2a) and blackish light green color callus in leaf (Figure 1a) explant was observed during the initiation of callus from *Muntingia calabura. Acacia auriculiformis*, *Strelitzia reginae*, and *Anemone coronaria* have also been reported that this problem can be prevented by adding up of activated charcoal (AC) to the medium which discolors and stimulates the shoot growth [YADAV & al. 2016]. Whereas the present work promoted by frequent subculturing of explants for every 10-20 days to reduce the browning of callus and the growth of callus kept increased.

Indirect regeneration studies from leaf explants. Regeneration of high number of shoots of *Muntingia calabura* with proper growth was achieved by testing varying combination of cytokinins (BAP and KN) with auxins (IAA and NAA). In leaf explants, 2 to 4 micro shoots were initiated from second week's brown or coffee colored callus (Figure 1b). After third-week brown colored callus slowly changed to green colored callus and few shoots formation was occurred simultaneously (Figure 1c). The leaf explants exhibited a highest shoot regeneration of 60% when cultured on multiplication medium with BAP (2.0 mg l^{-1}) or KN (2.0 mg l^{-1}) in combination with 0.5 mg l^{-1} IAA or 0.5 mg l^{-1} NAA. The explants inoculated onto the medium containing a low level of auxin and cytokinin have shown their response by early enlargement of nodes with axillary bud break within 25 days (Figure 1d). The proliferated shoot buds are shifted to a medium where the concentration of cytokinin is kept increased and the auxin concentration was maintained constant (Figure 1e). The number of shoots developed from leaf explants ranged from 13±0.3h to 32±0.8a on medium fortified with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} IAA (Figure 1f). The highest shoot length was found to be 7.6±0.1a on BAP (2 mg l^{-1}) and IAA (0.5 mg l^{-1}) MS medium (Figure 1g and 1h). The synergistic effect of BAP with IAA on shoot elongation has also reported in *Acacia auriculiformis* [YADAV & al. 2016]. An average of 4±06j to 30±0.6b (Table 1)

number of shoots were obtained on medium containing 2.5 mg l^{-1} KN with 0.5 mg l^{-1} IAA and 1±0.5k to 24±0.1e (Table 1) with 2.0 mg l^{-1} KN and 0.5 mg l^{-1} NAA in leaf explants. Excellent high frequency regeneration of *Populus deltoides*, a hardwood tree was also reported in combination of KN and IAA [YADAV& al. 2009]. Whereas, in *Gardenia latifolia* BA (4.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) had the best results for shoot regeneration from leaf [REDDY & SARITHA, 2013]. The hostile effect of cytokinin percentage in shoot induction decreases as the concentration of cytokinin increases. This effect has also been reported in *Pterocarpus marsupium* [ANIS & al. 2005]. The BAP is more efficient and more energetic cytokinin in many tree species like *Syzygium alternifolium* [SHA VALLI KHAN & al. 1999] and *Pterocarpus marsupium* [CHAND & SINGH, 2004]. Decisively, the combination of auxins and cytokinins in the medium can improve organogenesis and regeneration of the cultured explants.

Figure 1. Effect of PGRs on indirect plant regeneration from leaf explants: a – Initiation of callus on 2,4-D at 0.5 mg l^{-1}; b – Initiation of shoots in combination with 0.5 mg l^{-1} BAP + IAA 0.5 mg l^{-1}; c – Proliferation of micro-shoots at 0.5 mg l^{-1} IAA + BAP 1.0 mg l^{-1}; d – Elongation of shoots at 0.5 mg l^{-1} IAA + 1.0 mg l^{-1} BAP; e – Formation of multiple shoot clusters on 0.5 mg l^{-1} + IAA + 1.5 mg l^{-1} BAP; f – High frequency of multiple shoots formed on 2 mg l^{-1}; g & h – Elongation of shoot with roots at alone IBA 2.0 mg l^{-1}; i – Partially hardened plant in an earthen pot containing 1:1 vermiculite inside the greenhouse; j – Green house grown plants transferred into field.

Table 1. Effect of different concentrations of PGR's on multiple shoot induction using leaf and node explants of *Muntingia calabura* L. – a pharmaceutically and medicinally important woody tree.

PGR'S and Concentration				Regeneration		No. of shoots		Shoot length(cm)	
BAP	KN	IAA	NAA	Leaf	Node	Leaf	Node	Leaf	Node
0.5		0.5		67	61	13±0.3h	16±0.5cde	1.5±0.2h	1.7±0.1g
1		0.5		70	68	23±0.5e	16±0.8cde	2.8±0.1fg	3.7±0.1d
1.5		0.5		77	71	27±0.1c	20±0.4b	6.4±0.3b	9.3±0.4a
2		0.5		93	88	32±0.8a	26±0.8a	7.6±0.1a	9.6±0.3a
2.5		0.5		84	80	24±0.7de	18±0.6b	5.5±0.4c	6.9±0.3b
3		0.5		67	76	18±0.8f	10±0.1gh	3.0±0.3ef	3.6±0.2de
	0.5	0.5		46	40	4±0.6j	2±0.2jkl	0.5±0.2i	0.1±0.1i
	1	0.5		55	48	10±0.4i	8±0.3hi	1.4±0.3h	1±0.2hi
	1.5	0.5		55	50	23±0.5e	14±0.8f	5.1±0.4d	3.2±0.4def
	2	0.5		62	58	25±0.5cde	17±0.5cd	5.5±0.3cd	5.4±0.2c
	2.5	0.5		70	66	30±0.6b	25±0.6a	7.5±0.5a	6.3±0.3b
	3	0.5		68	58	25±0.8cde	16±0.4cdef	7.5±0.2a	3.5±0.2def
0.5			0.5	54	58	4±0.5j	1±0.3l	1.6±0.2h	0.5±0.2hi
1			0.5	67	60	10±0.5i	3±0.1jk	3.6±0.2e	1.5±0.2g
1.5			0.5	77	69	15±0.3gh	9±0.5ghi	3.6±0.2e	2.6±0.1f
2			0.5	80	75	26±0.2d	17±0.8cd	6.4±0.4b	3.6±0.2ef
2.5			0.5	78	64	16±0.2g	11±0.5b	2.4±0.3f	3.2±0.4def
3			0.5	60	50	10±0.4i	7±0.33i	1.5±0.2h	0.9±0.05i
	0.5		0.5	57	46	1±0.5k	0.6±0.33l	0.5±0.2i	0.2±0.2i
	1		0.5	66	60	5±0.5j	2±0.2jkl	2.0±0.2f	0.5±0.2hi
	1.5		0.5	70	55	19±0.6f	10±0.6gh	2.7±0.1ef	0.5±0.2hi
	2		0.5	75	64	24±0.1de	15±0.5ef	5.1±0.4c	2.7±0.1ef
	2.5		0.5	66	52	16±0.2g	9±0.3ghi	4.7±0.1d	2.7±0.1ef
	3		0.5	56	45	8±0.33i	3±0.5j	1.8±0.1h	1.3±0.4g

Direct regeneration studies from node explants. Development of new axillary shoots on medium with varying concentrations of BAP or KN in addition to IAA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) has revealed that BAP is efficient over KN in all the combinations. The combination of BAP 2 mg l^{-1} + IAA 0.5 mg l^{-1} is found to be efficient for shoot bud elongation in node than in leaf (Figure 2c). Similarly, the regeneration of the node had shown less response compared to leaf with BAP 2 mg l^{-1} in combination with NAA 0.5 mg l^{-1} and IAA 0.5 mg l^{-1}. Node explants showed maximum number of shoots from 16±0.5cde to 26±0.8a on BAP 2 mg l^{-1} + IAA 0.5 mg l^{-1} and from 1±0.3i to 17±0.8cd on BAP 2 mg l^{-1} + NAA 0.5 mg l^{-1} (Figure 2d and Table 1) and the highest shoot length of 9.6±0.3a was also developed on BAP 2 mg l^{-1} + IAA 0.5 mg l^{-1}. In nodal explants a range of 2±0.2jkl to 25±0.6a (Figure 2e and Table 1) mean number of shoots was noticed on medium with KN 2.5 mg l^{-1} and IAA 0.5 mg l^{-1} whereas 0.6±0.33i to 15±0.5ef number of shoots were developed with KN 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} g/l after 6 weeks. In *Artemisia nilagirica*, a high regeneration frequency occurred when nodal explants were cultured on MS medium with 2.5 mg l^{-1} BAP and 7.5 mg l^{-1} 2-iP [SHINDE & al. 2016]. In *Elaeocarpus* family, the species *Elaeocarpus sphaericus* and *Elaeocarpus robustus* an efficient micropropagation method using BA+KN+casein hydrolysate was reported from node explants. The best

results for shoot induction was reported in *Elaeocarpus sphaericus* [SAKLANI & al. 2015, LAKSHMI & al. 2010] on MS+2.2µM BA+2.2µM KN, whereas with equal concentrations of BA + KN + casein hydrolysate in *Elaeocarpus robustus* [ROY & al. 1998]. ARSHAD & KUMAR (2006) have also developed a protocol using axillary buds of *Elaeocarpus tuberculatus* on MS medium containing equal concentrations of only cytokinins in the combination of BA and KN.

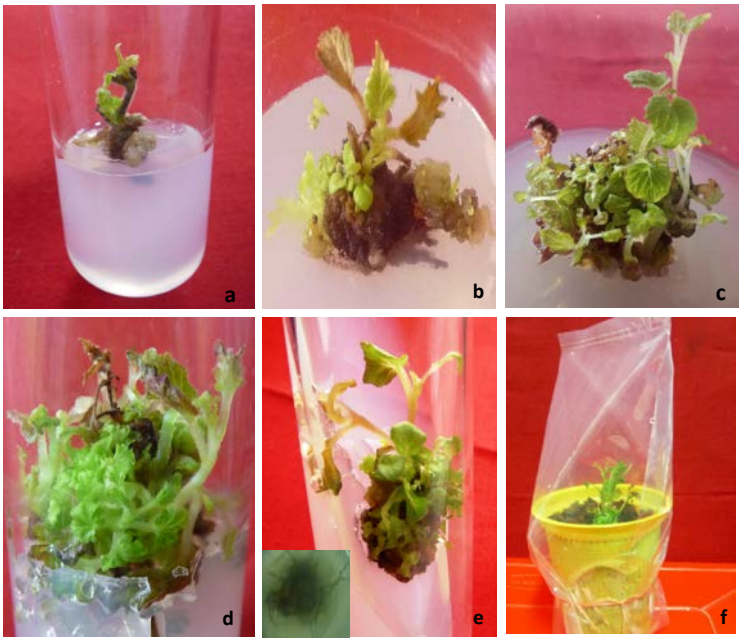

Figure 2. Effect of PGRs on direct regeneration from node explants: a – initiation of callus from mature nodal explants on MS medium with 0.5 mg l^{-1} 2,4-D; b – proliferation of shoots from nodal brown calli on MS medium with 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} IAA; c – elongation of multiple shoots with 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} IAA; d – elongation of multiple shoots with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} IAA; e – formation of roots from the lower part of the *in vitro* shoots on MS medium containing 2.0 mg l^{-1} IBA; f – *in vitro* regenerated plantlets transferred to plastic cup possessing soil and vermicompost (1:1) and the plantlets covered with polythene sheet for acclimatization.

Rooting for in vitro shoots. Individual shoots were excised and inoculated onto half-strength MS medium containing IBA. IBA at a concentration of 2.0 mg l^{-1} induced rooting with a root length of 2±0.33b with maximum 25±0.57a (Figure 2e-r and Table 2) number of roots. But the maximum root length of 3±0.3a was observed at IBA 2.5 mg l^{-1}. At lower concentrations of IBA, the number of roots grown was less comparatively at 2.0 mg l^{-1}. Interestingly, as the concentration of IBA is increased above 2 mg l^{-1} basal callusing was observed all through the surface of shoots and there was also a decrease in root length. IBA has been identified to induce the best rooting response in woody tree species. Similar reports were observed in *Gardenia latifolia* with IBA at 4.0 mg l^{-1} of concentration on half-strength MS medium [REDDY & SARITHA, 2013].

Table 2. Effect of different concentrations of IBA on rooting of *in vitro* regenerated shoots of *Muntingia calabura* after 6 weeks ½ strength MS medium.

Auxin IBA	% of rooting	Root number	Root length (cm)
0.5	20	5±0.33e	0.2±0.3d
1.0	39	12±0.57d	0.5±0.3cd
1.5	55	18±0.33c	1±0c
2.0	60	25±0.57a	2±0.2b
2.5	51	21±0.57b	3±0.3a

Hardening and acclimatization. *In vitro* grown complete plantlets of *Muntingia calabura* are carefully taken out and washed gently to remove the adhered media. Polythene cups containing garden soil and vermicompost in 1:1 ratio was found to be best for hardening (Figure 2f). Hardened plantlets were maintained initially in greenhouse conditions by irrigating with MS salt solution (half-strength) for four weeks (Figure 1i). The poly cups with hardened plantlets were covered with polythene sheets and proper watering was done to maintain humidity (50-70%) and moisture. Later, plantlets were planted and acclimatized in field conditions with high survivability of 85% was observed (Figure 1j).

Genetic fidelity analysis. The ISSR and RAPD markers were employed to identify the genetic fidelity of the mother plant and the regenerated plants of *Muntingia calabura*. Earlier reports suggested that using of more than one type of molecular marker in genetic homogeneity studies of *in vitro* regenerated plants is always useful for reliability [ROHELA & al. 2019]. In the current investigation, ISSR (10) and RAPD (10) primers (Table 3 & Table 4) were used for the screening of genetic homogeneity of *in vitro* regenerated plants with the parent plant. In out of 10 ISSR primers, seven primers were produced reproducible and clear bands, the PCR banding pattern of MC4 primer shown in Figure 3. In out of 10 RAPD-primers, six primers were amplified with scorable and clear reproducible bands, the PCR banding profile of OPA6 shown in Figure 4. ISSR and RAPD analysis confirmed that there is no polymorphic nature in regenerated and mother plants; both plants exhibited similar monomorphic banding patterns. Therefore, the results prove that the mother plant and regenerated plant contains the same gene pool (Figure 3, 4). A similar kind of genetic fidelity evaluation using ISSR and RAPD markers was successfully achieved in different plant systems like *Rauwolfia tetraphylla* [ROHELA & al. 2019], *Citrullus colocynthis* [RAMAKRISHNA & al. 2012], *Solanum trilobatum* [PENDLI & al. 2019], and *Flemingia macrophylla* [SIRIKONDA & al. 2020].

Table 3. List of ISSR and RAPD primers used in genetic fidelity analysis of *Muntingia calabura* L. – a pharmaceutically and medicinally important woody tree.

ISSR			RAPD		
S.No.	Primer code	Primer sequence (5'-3')	S.No.	Primer code	Primer sequence (5'-3')
1	MC1	TCTCTCTCTCTCTCTCC	1	OPA1	TGGGCGTCAA
2	MC2	TCTCTCTCTCTCTCTCG	2	OPA2	CAGGCCCTTC
3	MC3	AGAGAGAGAGAGAGAGC	3	OPA3	GGCATGACCT
4	MC4	AGAGAGAGAGAGAGAGG	4	OPA4	GGGTAACGCC
5	MC5	GAGAGAGAGAGAGAGAC	5	OPA5	CCAGCAGCTT
6	MC6	GAGAGAGAGAGAGAGAT	6	OPA6	AGTCAGCCAC
7	MC7	GAGAGAGAGAGAGAGAYT	7	OPA7	TGCCGAGCTG
8	MC8	AGAGAGAGAGAGAGAGYT	8	OPA8	AATCGGGCTG
9	MC9	GAGAGAGAGAGAGAGAYG	9	OPA9	GACTGCACAC
10	MC10	AGAGAGAGAGAGAGAGTC	10	OPA10	AGGGGTCTTG

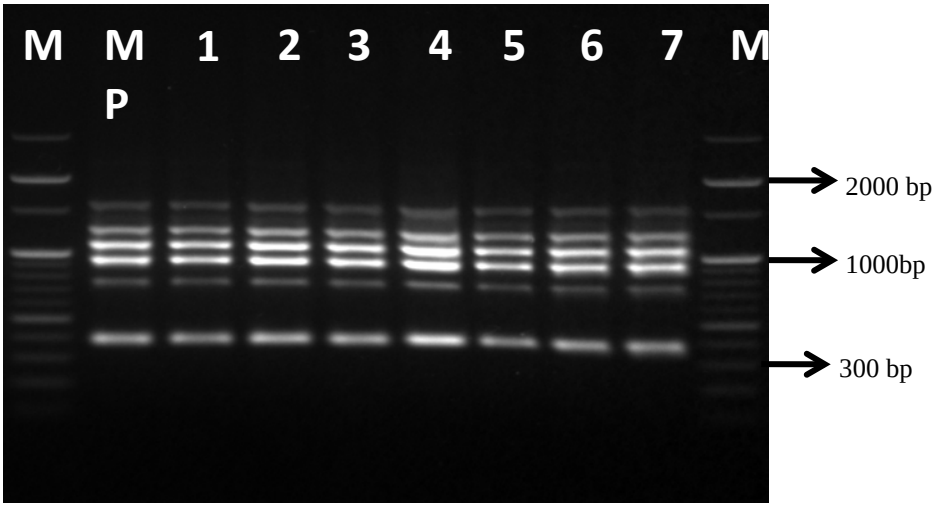

Figure 3. PCR banding profile of ISSR based genetic fidelity analysis of *Muntingia calabura* amplified with MC4 primer. Lane M: 100 bp DNA ladder (Himedia, India). Lane MP: PCR banding pattern of mother plant. Lane 1–7: PCR banding pattern of regenerated plants.

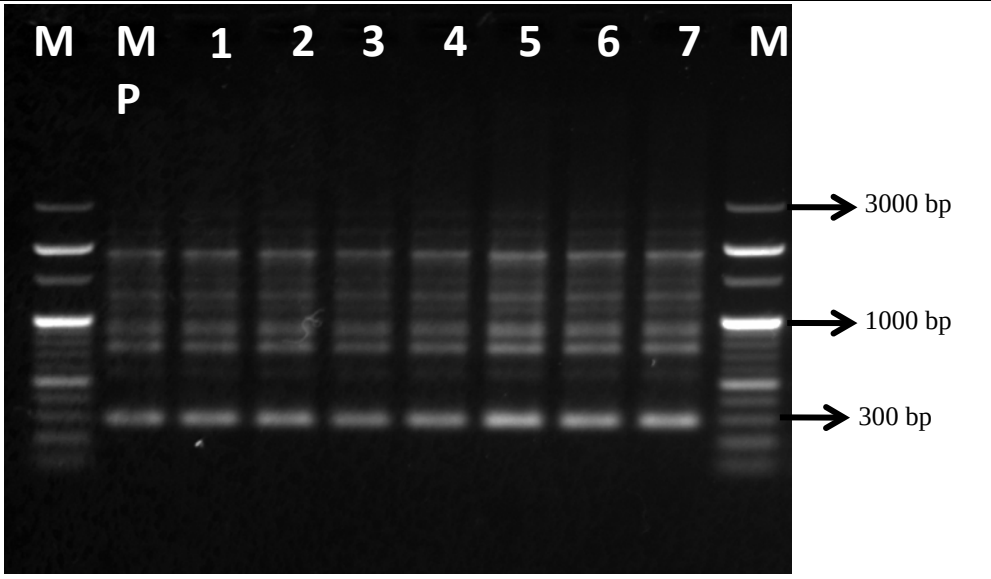

Figure 4. PCR banding profile of RAPD based genetic fidelity analysis of *Muntingia calabura* amplified with OPA6 primer. Lane M: 100 bp DNA ladder (Himedia, India). Lane MP: PCR banding pattern of mother plant. Lane 1–7: PCR banding pattern of regenerated plants.

Conclusions

The present investigation appears to be the first report on regeneration studies of medicinally important woody tree *Muntingia calabura* and assessment of genetic fidelity demonstrates the true-to-type nature of regenerated plants comparatively to the mother plant. Our research findings established the protocol of regeneration studies in the woody tree using PGRs at lower concentrations with eradicating the problem of browning callus without using any anti-browning agents through regular subculturing of cultures. The highest number of shoots was observed to be 32±0.8a in leaf explants on MS medium along with BAP 2.0 mg l^{-1} + IAA 0.5 mg l^{-1} and 26±0.8a in node explants on BAP 2.0 mg l^{-1} + IAA 0.5 mg l^{-1}. The highest rooting frequency is 25±0.57a was obtained on IBA 2.0 mg l^{-1}. The regeneration in leaf and node explants cultured on to MS medium with BAP or KN in combination with IAA and NAA exposed that BAP is more efficient to KN in all tried combinations. BAP has given the maximum efficiency of shoots comparative to KN, with the addition of IAA produced a high organogenic response of the explants. Further, *in vitro* regenerated acclimatized plantlets are using for isolating and screening of secondary metabolites from various plant parts. For the above reasons, the biotechnological analysis studies are required in *Muntingia calabura*.

Notes on contributors

Suvarchala VANKUDOTH is PhD student and she has conducted the total experimental work.
Ramakrishna DASARI is a research scientist and expert in plant tissue culture and Biotechnology.
Pavani CHIRUMAMILLA is a PhD student and she has conducted the total experimental work.
Chaitanya GOPU is also a PhD student and she has designed the figures and tables.
Phanikanth JOGAM is a PhD completed student and he has conducted genetic fidelity study.
Srinivas KOTA is a PhD student and he has edited the whole manuscript.

Shasthree TADURI is a senior grade professor and plant biotechnologist. His work focuses on developing regeneration protocols for the medicinal and endangered plants. His research group is actively involving in isolation, screenings, and enhancement of bioactive compounds using biotic and abiotic elicitors. Shasthree TADURI availed Dr. CV Raman Fellowship in Mississippi State University, USA and Associated fellow of Telangana Academy of Sciences. He also attended and presented his research findings in national and international conferences.

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PHYTOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL CHARACTERIZATION OF *HEDERA HELIX* L. EXTRACT

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Abstract: The present research describes the components of the ivy (*Hedera helix* L.) plant extract. It is known that ivy presents a lot of health benefits, like antibacterial, antimicrobial, anticancer properties or skin care, due to their components. So, the aim of our study was to characterize quantitative (polyphenols, tannins, flavonoids, terpenoids) and qualitative (saponins, proteins, steroids) screening for phytochemical compounds and antioxidant activity of the hydroalcoholic extract obtained by ivy leaves. The sample was analyzed by UV-VIS, FTIR, TLC techniques. The antioxidant activity was evaluated using DPPH method. The antimicrobial activity was demonstrated on bacteria, yeast and mold species.

Keywords: analytical techniques, antioxidant activity, *Hedera helix* L., phytocompounds, phytosynthesis.

Introduction

Ivy (*Hedera helix* L.), is a genus containing approximately 15 species of climbing evergreen plants. On suitable surfaces, trees and rock faces, ivy has the ability to climb 25-30 m above the ground and hold fast to vertical surfaces, like liana [XIA & al. 2011; LUTSENKO & al. 2010].

Hedera helix contains saponins, flavonoids, polyacetylenes and phenolic compounds (flavonoids, anthocyanins, coumarins and phenolic acids), aminoacids, steroids, vitamins, volatile and fixed oils, which are reported for medicinal benefits: antifungal, antispasmodic, antimicrobial, antimutagenic or cytotoxic activities [BUSECK & al. 2011; MEDEIROS & al. 2002; MIAO & al. 2015].

As we said above, the main constituents in the crude extract ivy plant are triterpen saponins, with the predominant substance Hederacozide C, presented in Figure 1.

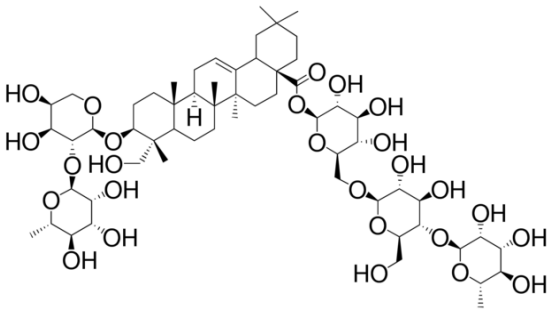

Figure 1. Structure formula of hederacozide C.

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Material and methods

Ivy plant (*Hedera helix* L.) was collected from ICECHIM institute garden, Bucharest city. All solvents used for extraction were of analytical grade. Methanol was purchased from Merck. Distilled water was internal laboratory obtained, using Liston equipment. For determinations of phytochemical methods, it was used $AlCl_3$ (from Sigma-Aldrich), $NaNO_2$, NaOH, H_2SO_4, Na_2CO_3, HCl and Folin–Ciocalteu reagent (from Merck) substances. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate stable free radical, Merk) was utilized for antioxidant determination. For qualitative determinations, Benedict's and Millon's reagents were purchased from Sigma – Aldrich.

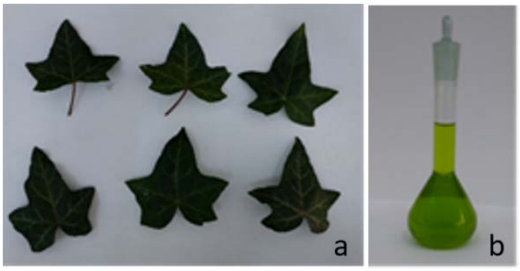


Figure 2. a. Ivy leavesand b. hydroalcoholic ivy extract.

The ivy leaves (Figure 2a) were washed and dried at room temperature, 5 days. These where extracted in a mixed solution (ethanol: distilled water) using an ultrasonicator bath (50 ºC, 90 min, 100 power). After that, the extract was kept 6 days at maceration, at room temperature. Then it was filtered and the final solution (Figure 2b) was kept in the fridge, in order to avoid carotenoid degradation.

UV-VIS Spectroscopy. The absorption spectra of the samples were recorded on a double beam M400 Carl Zeiss Jena UV-VIS spectrophotometer from 250 to 750 nm, at the resolution of 1 nm, with 1 nm slit width and 0.3 nm/s scan rate.

FTIR Spectroscopy. For Fourier transformed IR spectroscopy, the spectra were collected using a Perkin Elmer Spectrum GX instrument. Spectra were registered using ATR technique, in the range of 4000-600 cm^{-1} at a spectral resolution of 4 cm^{-1}.

Antioxidant activity (*AA%*). The principle of AA% method consists in reducing the presence of an antioxidant molecule, giving rise to colored methanol solutions. The utilization of DPPH method gives an easy and rapid result to antioxidant activity against free radicals [SUICA-BUNGHEZ & al. 2020].

Phytochemical analyses. The phytochemical analyses were used for the determination of total tannins, total flavonoids, total polyphenols, total terpenoids and carotenoids existent in the hydroalcoholic ivy extract.

Qualitative screening for phytochemical compounds. The qualitative screening refers mainly to the change in color of the aqueous ivy (*Hedera helix* L.) extract when known reagents are added, indicating the presence or absence of different phytochemicals such as carbohydrates, tannins, saponins, proteins, alkaloids and glycosides.

TLC method. It was used to observe the presence of β-carotene, chlorophylls A and B pigments, in the ivy extract sample. To achieve thin layer chromatography, some specific steps for this technique, was followed. The ivy extract sample was evaporated to concentrate

the pigments. The chromatographic tank was prepared, using a solvent mixture: hexane 70%: acetone 30%. The samples were spotted on the silica gel plate, and this was introduced into the chromatographic tank. The compound elution was stopped when it reached 2 cm from top of the plate. The spots formatted on the chromatograms, were visualized with the use of a UV lamp. The final step was to calculate R_f (retention factor) values, utilizing the equation:

$$R_f = \text{distance spot moved/distance solvent moved}$$

Antimicrobial activity. The in vitro antimicrobial activity of the extract was evaluated by the disk-diffusion and agar dilution methods. For the disk-diffusion technique the agar plates were inoculated with standardized inoculums of the test microorganism equivalent to the 0.5 McFarland standards by streaking, and after that the paper discs (about 6 mm in diameter) were placed on the agar and inoculated with 20 µl of the extract. The Petri plates were placed in incubator for 24 hours, at 35 °C for bacteria and 28 °C for the fungi. Following the incubation time, the inhibition zone sizes were measure using a ruler. In the agar dilution test, 20 µl of the extract was used by spotted on the agar: Mueller Hinton Agar for bacteria and Sabouraud for the fungal strains. The ivy extract sample was tested on *Escherichia coli* and *Staphylococcus aureus* bacteria, *Candida albicans* yeast and *Aspergillus niger* mold.

Results and discussions

The components and phytosynthesis of extracts were confirmed by modern analytical techniques (UV-VIS and FTIR spectroscopy) and by TLC (thin layer chromatography) method

UV-VIS results. UV-visible spectroscopy was used to characterize the hydroalcoholic ivy extract (Figure 3). The wavelength spectrum was registered between 250-750 nm. It was identified the wavelengths specific to flavonoids and phenolic acids at 300-350 nm. Another peak appears between 400-420 nm specific to carotenoids, the peaks between 420-460 nm and 600-650 nm are specific to chlorophyll B and the peak at 660 nm it is characteristic for chlorophyll A [LICHTENTHALER, 1987; BRITTON, 1995].

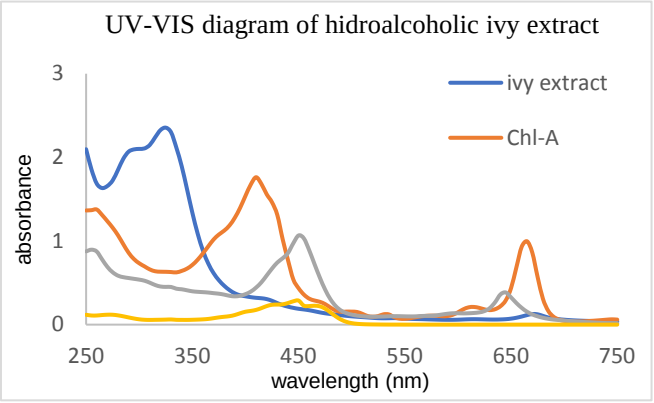


Figure 3. UV-VIS spectra of ivy leaves extract.

FTIR results. The infrared spectral analysis was carried out to characterize the type of functional group existent in ivy leaves extract. FTIR spectra (Figure 4) of the different phytocompounds in ivy extract were recorded to observe the functional groups contained in this type of plant. The spectrum (Figure 3) was recorded between 4000-600 cm^{-1}. The bands C–N, C=C and C=O are found at 1600 cm^{-1} region. The CO groups, from esters, hydroxy flavones, catechins and type II amides and the C–H bending aliphatic amine functional groups are predominant in the range of 1268-1206 cm^{-1}. The peak at 1045 cm^{-1} was associated with vibration of the CO–C bond typically found in carbohydrates.

The ivy extract exhibit weak IR bands between 814-643 cm^{-1} specific for C-N stretching vibrations of aliphatic amines or C-O stretching vibrations of alcohols or phenols, which are found due to different types of phytocomponents present in the fruit extract (polyphenols, polysaccharides and proteins) [SUICA-BUNGHEZ & ION, 2017].

At 1693 cm^{-1} are found the COO- functional groups. The peaks around 1600 cm^{-1} and 1449 cm^{-1} represents the amide II bands and these are standard protein peaks, indicating the presence of N–H in-plane bending, and the stretching vibrations of C–N and C–C. The broad band present at 3330 cm^{-1} in the ivy sample indicates that both O–H bonds and N–H vibration were present [HEMMALAKSHMI & al. 2017].

Alkanes are characterized by stretching and bending vibrations of C-H groups at 2927 cm^{-1}. The aromatic group of the amide of type I and II are observed in the region between 1387 and 1365 cm^{-1}. A narrow adsorption band at 1660–1693 cm^{-1} could be assigned to carbonyl group. Moreover, stretching vibrations of C-O groups can be detected at 1030 cm^{-1}. The presence of stretching vibrations of C=O groups suggest the structure of oleanane-type triterpenoid saponins, most likely bidesmosides [ZDARTA & al. 2019; SCHULZ & al. 2005].

At 2927 cm^{-1} are identified asymmetric stretching of –CH (CH_2) vibration. The peak around 2275 cm^{-1} is characteristic to C-C triple bond. Between 1990-1938 cm^{-1} appears carbonyl compound frequency [AROCKIA SAHAYARAJ & al. 2015].

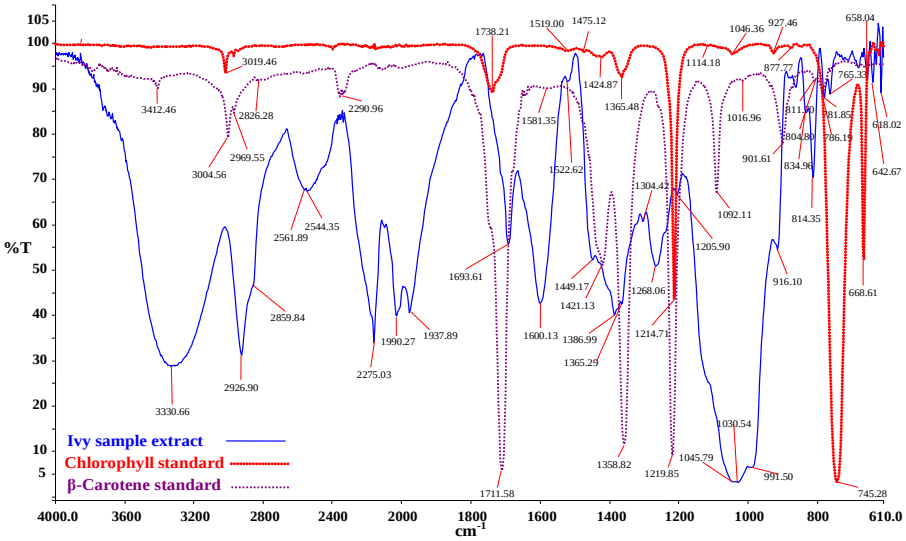


Figure 4. FTIR spectrum of ivy extract sample, chlorophyll and beta-carotene standards.

Phytochemical results are presented in Table 1.

Table 1. Phytochemical results of ivy extract sample.

Assays	Ivy extract results
Total tannins	13.5 mg/L
Total flavonoids	212.5 mg/L
Total terpenoids	107.77 mg/L
Total polyphenols	779.66 mg/L
Total carotenoids	2.586 mg/mL
Antioxidant activity	75.88%

Qualitative phytochemical results (Table 2), related the presence of saponins, tanins and terpenoids in the ivy extract sample.

Table 2. Qualitative phytochemical results of ivy extract sample.

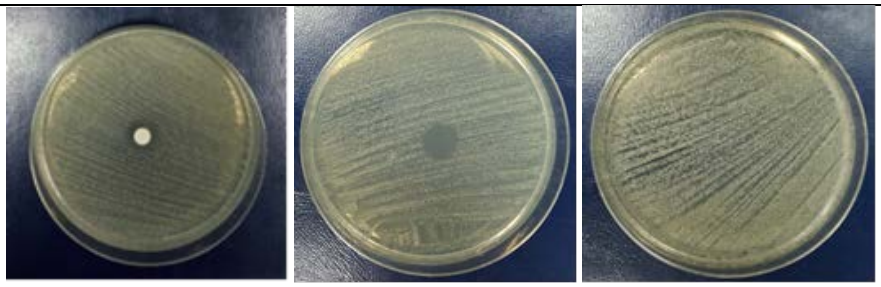
Phytochemical test	Ivy extract results (+) presence; (-) absence
Saponins	+
Tannins	+
Proteins and aminoacids (Millon's test)	-
Protein and aminoacids (copper sulphate test)	-
Steroids	-
Terpenoids	+

Thin layer chromatography (*TLC*) *analysis of the ivy extract.* The solvent mixture introduced in chromatographic tank was C_6H_{14}:C_3H_6O (70%:30%). Using a UV lamp, the spots migrated on the chromatogram were observed at 366 nm wavelength. The TLC results are represented in Table 3.

Table 3. R_f results of ivy extract sample and standards (β-carotene, chlorophyll A and B).

Sample	R_f value
Chlorophyll A	0.905
Chlorophyll B	0.908
β-carotene	0.930
Ivy extract	0.918

Antimicrobial activity. The antimicrobial activity of plant extracts is due to chemical constituents, such as alkaloids, polyphenols, saponins and essential oils [GAZDARU & ION 1994]. One of the aim of this research was to highlight the in vitro anti-bacterial and anti-fungal potential of phenolic extracts and compounds from the ivy extract. A qualitative method it was used, using blank, disc diffusion and spot. The ivy extract sample was tested on bacteria, yeast and mold. No antimicrobial activity on *Escherichia coli*, *Staphylococcus aureus* bacteria was observed. On *Aspergillus niger* mold, it was saw a slight reduction in growth, which led to the conclusion that a more concentrated sample could have a better effect. But for *Candida albicans* yeast, it was observed a good antimicrobial activity.



a. disc diffusion b. spot c. blank

Figure 5. The antimicrobial activity of the *Hedera helix* leaf extract on *Candida albicans*, a. disc diffusion, b. spot and c. blank.

Conclusions

In the present study were determinated total flavonoids, polyphenols, terpenoids, tannins, carotenoids and antioxidant activity of ivy (*Hedera helix* L.) plant. Also, the sample extract compounds were characterized using different types of analytical methods (FTIR, UV-VIS). The existence of phenolic compounds in the ivy leaves was confirmed by the Folin-Ciocalteu method. ATR-FTIR results demonstrated the major amount of carbohydrates, aminoacids, proteins, phytoingredients, hydroxyl functional groups (polyphenols) and chlorophyll and carotene pigments. TLC, also demonstrated the presence of photosynthetic pigments in ivy extract. The antioxidant capacity was measured by the free radical scavenging methods DPPH. The methanolic solutions of the ivy extract showed high antioxidant capacity (AA = 75.88%). All results of phytochemical analyses were made in triplicate and calculated using calibration curves results, with very good regression indices. The *Hedera helix* L. extract sample was tested on *Escherichia coli* and *Staphylococcus aureus* bacteria, *Candida albicans* yeast and *Aspergillus niger* mold. It was observed a law activity on *A. niger* and a better activity on *C. albicans.*

Notes on contributors

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PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITIES OF *VERNONIA AMYGDALINA* (BITTER LEAF), *TELFAIRIA OCCIDENTALIS* (PUMPKIN LEAF) AND *OCIMUM GRATISSIMUM* (SCENT LEAF)

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Abstract: Phytochemical and antimicrobial screening is the extraction, analysis and identification of the bio-active components found in plants and the effects of such bio-active components against bacterial species. The analysis were conducted on three plant samples: *Vernonia amygdalina* (Bitter leaf), *Telfairia occidentalis* (Pumpkin leaf) and *Ocimum gratissimum* (Scent leaf). The leaf samples were collected from the Botanical Garden of the department of Biological Sciences Ibrahim Badamasi Babangida University Lapai, Niger State, Nigeria. The bacterial isolate; *Staphylococcus aureus* was collected from the department of Microbiology, Ibrahim Badamasi Babangida University, Lapai, Niger State, Nigeria. The phytochemical screening of the plant samples was conducted using standard methods, where bio-active components such as saponins, tannins, flavonoids, alkaloids, phenols, glycosides, phlobatannins and steroids were screened. The antimicrobial analysis was conducted using the Nutrient agar diffusion method. The bio-active components found from the phytochemical screening were saponins, tannins, glycosides, alkaloids and flavonoids. The antimicrobial activities showed the highest zones of inhibition against *Staphylococcus aureus* in *Vernonia amygdalina* (7.5 mm) and lowest in *Ocimum gratissimum* (6.0 mm). These leaf samples are good source of bio-active components which are capable of suppressing bacterial infection that cause diseases.

Keywords: Antimicrobial screening, bio-active components, *Ocimum gratissimum*, *Telfairia occidentalis*, *Vernonia amygdalina.*

Introduction

Phytochemical screening is the extraction, qualitative analysis and identification of the medicinally bio-active substances found in plants [YESSUF, 2015]. Many reports by [ONIFADE & AGUNLOYE, 2019; OBOH, 2006; EVBUOMWAN & al. 2018] have reported that the bioactive substances found in *Vernonia amygdalina* Delile (Family Compositae), *Telfairia occidentalis* Hook.f. (Family Cucurbitaceae) and *Ocimum gratissimum* L. (Family Lamiaceae) are flavonoids, alkaloids, tannin, saponin, phenolics and antioxidants. Thus, it can be therefore concluded that *V. amygdalina*, *T. occidentalis* and *O. gratissimum* leaves can greatly contribute to the nutrient requirements of man and should be consumed as they great sources of minerals and vitamins to supplement other major sources.

Although the knowledge of how these bio-active substances provide medicinal benefits to humans reflects the scientific understanding, the exploitation of plants and plant extracts to treat, relieve pain and improve good health dates back to before the invention of medical science [SOFOWORA, 1999; UDOCHUKWU & al. 2015]. Reports has shown that there are about 4000 phytochemicals that are found in plants, and these phytochemicals are

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capable of providing medical remedies such as strokes, cancer and microbial infections [YEDJOU & al. 2008; EVBUOMWAN & al. 2018].

Many of the local vegetable are widely consumed not just for their nutritional or phytochemical potentials, but for their ability to fight against diseases or infections that are caused by certain microorganisms. Many studies [SAXENA & SAXENA, 2002; IBRAHIM & al. 2009] have reported that leaves of *V. amygdalina*, *T. occidentalis* and *O. gratissimum* has an effective antimicrobial activities on bacteria such as *Escherichia coli* and *Staphylococcus aureus*.

Many plants have been used because of their antimicrobial properties; this is due to the bio-active compounds present which are synthesized in the secondary metabolism of the plants. These plants are known by their bio-active substances, for example; phenols are compounds which are components of the essential oils, as well as in tannin [DAS & al. 2010].

Vernonia amygdalina, Telfairia occidentalis and *Ocimum gratissimum* are vegetable crops are considered as weeds on fields and farm lands [OGWU & al. 2016]. *V. amygdalina, T. occidentalis* and *O. gratissimum,* have various phytochemical constituents and are capable of inhabiting against microorganisms such as bacterial species. This study was based on the phytochemical screening of *V. amygdalina, T. occidentalis and O. gratissimum,* and their anti-microbial activities against *Staphylococcus aureus*.

Materials and method

Samples collection

Leave samples of *V. amygdalina, T. occidentalis* and *O. gratissimum* used were collected from the Agricultural Research Farm of the Faculty of Agricultural Lapai, Niger State. The collected samples were sealed and labeled separately in sterile polythene bags and were taken to the Laboratory of the Departments of Microbiology and Biochemistry of Ibrahim Badamasi Babangida University, Lapai.

Phytochemical screening of the plant samples

Collected samples were rinsed with distilled water and their stalks were removed and air-dried for 2-3 days at room temperature (25 °C) on a clean or laboratory bench. The air-dried samples were grounded into powdered form using pestle and mortar. The powdered samples were sieved with a 2.0 mm mesh sieve to obtain fine powdered sample. 30 ml of ethanol was added to the dried powdered samples and were properly mixed. The mixtures were filtered and the filtrates were kept for 2 hours for the ethanol to volatilize to obtain ethanolic extracts [YADAV & al. 2014].

The recommended method of Pharmaceutical and Allied Sciences [UGWOKE & EZUGWE, 2010] was used for the determination of saponins, alkaloids, tannins, phenols, flavonoids, steroid, glycosides and phlobatannins.

Antimicrobial screening of the bacterial isolates

The bacterial isolates used for the screening was *Staphylococcus aureus.* It was collected from the Department of Microbiology of Ibrahim Badamasi Babangida University, Lapai. The bacterial specie used was cultured on nutrients agar slant and was kept in the refrigerator at a temperature of 4 °C from which they were sub-cultured unto freshly prepared media at regular intervals.

Pure culture of *Staphylococcus aureus* was obtained by sub-culturing discrete colonies into freshly prepared nutrient broth and was incubated at 37 °C for 18-24 hours. The isolates developed were pure cultures which were stored in the refrigerator as stock culture for subsequent characterization [EVBUOMWAN & al. 2018].

Reconstitution and sterilization of extracts

The dried fine powdered leaves samples were measured into McCartney bottles and appropriate volumes of the extractants were added to obtain a stock solution of 200 mg/ml. Sterilization of the stock solution was carried out using 0.65 membrane filter by suction pump. The sterilized extract from the leaves were kept inside sterile McCartney bottle and stored in the refrigerator at 7 °C which was used for the antibacterial test. Test for sterility of the extracts investigated by placing it on nutrient agar and incubated for 24 hours at 37 °C [EVBUOMWAN & al. 2018].

Agar diffusion analysis of antimicrobial activity of the plant extracts

Sterilized nutrient agar were prepared, poured in a sterile culture plate and was kept to solidify. *Staphylococcus aureus* isolate of 0.1 ml of a day old was introduced into the plate and sterile cotton swab was used to spread the inoculant evenly on the surface of the agar and the excess was drained off. The plates were left on the bench for 1 hour for proper diffusion of the inoculant into the agar. Five dishes was bore on the plates using 5 mm sterile cork borer. Varying concentrations of the extracts which include 200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml were prepared and 0.5 ml of the extracts was dropped in each of the appropriately labeled ditch in the agar plate.

Control was set up for each plate and this was done by adding 0.5 ml of the appropriate extract into the 5^{th} ditch. The plates were triplicated and kept on the bench for few minutes for proper diffusion of the extracts into the agar and later were incubated at 37 °C for 24 hours. After incubation, the zone of clearance around each ditch was carefully measured using a metric ruler. This was done by taking measurement from the edge of the plate to the point where the growth of the organisms started. Measurement in diameter (mm) of the zone of inhibition against the tested microorganisms [GUPTA & al. 2016].

Results

Phytochemical analysis of the ethanolic and aqueous extracts of *Vernonia amygdalina* (Bitter leaf), *Telfairia occidentalis* (Pumpkin leaf) and *Ocimum gratissimum* (Scent leaf) leaf.

Phytochemicals such as saponins, glycosides, tannins, alkaloids, and flavonoids were all present in the ethanolic extracts of *V. amygdalina*, *T. occidentalis* and *O. gratissimum*. Phlobatannins was absent in all the three plant extracts. Steroids and phenolics was either present or absent in the three plant extracts (Table 1).

Saponins, tannins and alkaloids were all present in the aqueous extracts of *V. amygdalina*, *T. occidentalis* and *O. gratissimum*. Glycosides, flavonoids, steroid and phenolics were either present or absent in the three plant extracts. Phlobatannins was absent in the three extracts (Table 2).

Table 1. Phytochemical analysis of the ethanolic extract of *V. amygdalina*, *T. occidentalis* and *O. gratissimum.*

PHYTOCHEMICALS	*V. amygdalina*	*O. gratissimum*	*T. occidentalis*
Saponins	+	+	+
Glycosides	+	+	+
Tannins	+	+	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Phlobatannins	–	–	–
Steroids	–	–	+
Phenolics	+	–	+

Key: + = present; – = absent

Table 2. Phytochemical analysis of the aqueous extract of *V. amygdalina*, *T. occidentalis* and *O. gratissimum.*

PHYTOCHEMICALS	*V. amygdalina*	*T. occidentalis*	*O. gratissimum*
Saponins	+	+	+
Glycosides	+	–	–
Tannins	+	+	+
Alkaloids	+	+	+
Flavonoids	–	+	+
Phlobatannins	–	–	–
Steroids	–	–	+
Phenolics	–	–	+

Key: + = present; – = absent

Antimicrobial activities of the ethanolic and aqueous plant extracts against *Staphylococcus aureus*

The antimicrobial activity of *Vernonia amygdalina*, *Telfairia occidentalis* and *Ocimum gratissimum* (Table 3). The result revealed that the ethanolic extract of *V. amygdalina* at the concentration of 200 ml had the highest zone of inhibition of 7.5 mm while the least was observed at 6.0 m for *O. gratissimum*.

Aqueous extract of *V. amygdalina* had the highest zone of inhibition 6.0 mm at the concentration of 200 ml, while the least was observed at 4.0 mm for *T. occidentalis* (Table 3).

Table 3. Antimicrobial activity of *V. amygdalina*, *T. occidentalis* and *O. gratissimum* against *Staphylococcus aureus.*

Plant samples	Concentrations							
	Ethanolic extract (ml/mm)				Aqueous extract (ml/mm)			
	25	50	100	200	25	50	100	200
V. amygdalina	3.0	5.0	6.5	7.5	3.0	4.0	5.0	6.0
T. occidentalis	2.5	4.5	6.0	6.5	1.0	2.0	3.0	4.0
O. gratissimum	3.5	4.0	5.0	6.0	3.5	3.0	3.5	4.5

Discussion

Phytochemical analysis revealed the leaf extracts (aqueous and ethanolic) of *Vernonia amygdalina* (Bitter leaf), *Telfairia occidentalis* (Pumpkin leaf), and *Ocimum gratissimum* (Scent leaf) have the presence of saponins, tannins, alkaloids, flavonoids and glycosides. This findings correlate with the reports of [IZEVBIGIE & al. 2004, EVBUOMWAN & al. 2018] that leafy vegetables such as *V. amygdalina* had the presence of alkaloids, saponins, tannins and glycosides, antihelmitic, antimalarial, antitumourigenic, hypoglycemic and hypolipidaemic properties. Medicinal plants contain substances which could be used for treatment purposes and for drugs productions. Secondary active compounds such as tannins, saponins, glycosides and alkaloids were reported by UDOCHUKWU & al. (2015) to be present in leafy vegetables.

Many of these plants are known to be useful to alleviate symptoms of illnesses, and have been screened to have medicinal benefits, some of which are; *Azadirachta indica* (neem), *V. amygdalina* (Bitter leaf), *Allium sativum* (Garlic), *O. gratissimum* (Scent leaf), and *Zingiber officinale* (Ginger). These plants have been reportedly used in the treatment of ailments such as stomach disorder, fever symptoms and cough traditionally [YEDJOU & al. 2008, EVBUOMWAN & al. 2018].

The antimicrobial analysis of the leaf extracts of *Vernonia amygdalina*, *Telfairia occidentalis*, and *Ocimum gratissimum* showed profound antimicrobial activities against *Staphylococcus aureus*. The result revealed that there were differences in the degree of antimicrobial activities of the extracts. This is in accordance to the report of PELCZAR & al. (1993) the reported that the differences in the susceptibility of bacteria to various antimicrobial agents may be as a result of their structural differences in their cell wall. The obvious difference of the leaf extracts on *Staphylococcus aureus* therefore, is suggestive of the activities against the cell wall components of the bacteria isolate. The antimicrobial substances appear to exert antimicrobial activity by inhibiting the growth of and by killing the sensitive bacteria. The ethanolic extract of the plants have the highest zone of inhibition in all, this is definitely due to the higher concentrations of the bio-active components (saponins, tannins, alkaloids, flavonoids, and glycosides). This is in correlation to the work of IJEH & ADEDOKUN (2006). One of the factors that affect antimicrobial screening is the concentration of the bio-active components. The higher the concentration of the bio-active components, the higher the activities of the bio-active components.

The phytochemical and antimicrobial activities of *V. amygdalina*, *T. occidentalis* and *O. gratissimum* been demonstrated by OGUNDARE & ADEMOLA (2011) that the efficacy of these plants is as a result of the age of the plant, solvent use for the extraction, method used for extraction and the season of harvest of the plant materials.

Conclusion

The extracts of *Vernonia amygdalina*, *Telfairia occidentalis* and *Ocimum gratissimum* plants contain bio-active phytochemical substances and antimicrobial properties which are capable of inhibiting microorganisms to caused diseases in plants and animals.

Notes on contributors

Dr. Habiba Maikudi MUHAMMED is a plant biologist with special interest in plant pathology and plant physiology. Her focuses in the plant diseases and management methods of vegetable crops North central Nigeria.

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ALLELOPATHIC POTENTIAL OF *CAPSICUM ANNUUM* L. AND *CORIANDRUM SATIVUM* L. ON GROWTH OF BEAN CROP

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Abstract: Allelochemical are natural compounds which effects the growth of surrounding plants. The treatment of aqueous powder extracts of *Capsicum annuum* L. and *Coriandrum sativum* L. at 1% showed significant decreased in shoot, root and seedling height, leaves growth and biomass of mung bean (*Vigna radiata*). The inhibitory effect on growth performance was directly related with the increasing concentration of powder extracts of coriander. The seedlings of both legume bean were tested for the determination of toxicity and tolerance to red chilli and coriander powder extract treatment at 0, 1, 2, 3, 4 and 5%. The seedlings of mung bean and cowpea showed lowest percentage of tolerance indices to coriander and red chilli powder extract treatment at 5%. However, on comparison, the seedlings of cowpea showed more tolerance to coriander and red chili powder extract than mung bean.

Keywords: coriander, phytotoxicity, plant extracts, red chili, root growth, seedling growth, tolerance indices.

Introduction

The discharge of phytochemical substances from one plant species altered the growth performances of surrounding plants. Many studies have shown that the release of toxic substances by a plant decreased or promote growth growth of neighboring plants [CHOU & LEE, 1991; KIL & YUN, 1992; REINHARDT & al. 1993; ALLOLI & NARAYANREDDY, 2000; DAIZY & al. 2001; FERGUSON & RATHINASABPATHI, 2003; OUSSAMA, 2003; DONGRE & YADAV, 2005; MISHRA & al. 2014] and termed as allelopathy. The impact of leaf leachates of some woody plant species on agriculture and some desertt plants were reported [MELKANIA, 1984; HEGAZY & al. 1990; PURI & KHARA, 1991]. Seed regeneration failures of *Pinus silvestris* L. by *Empetrum hermaphroditum* Hagerup occurred due to allelopathic interference [ZACKRISSON & NILSSON, 1992].

Allelopathy helps in ecosystems management [ABBASSI & al. 2013] and some work in earlies 1970 was initiated in Pakistan [CHEEMA & al. 2013]. These chemicals products can serve for weed control [BAGHERI & al. 2013; BOJOVIĆ & JAKOVLJEVIĆ, 2015]. The release of allelopathic compounds influence positively and negatively on the growth of plants. *Capsicum annuum* L. belongs to Solanaceae family. *Coriandrum sativum* L. (Apiaceae) is popular medicinal plant and seeds are source of iron, copper, calcium, magnesium and zinc. Both spices production have great economic demands and used in cooking on daily basis in Pakistan and worldwide.

The purpose of the studies was to assess the toxic potential of red chili and coriander aqueous powders extract on the growth of two different bean crops namely, cowpea and mung bean because of economic importance of both leguminous crops are cultivating in the larger agricultural area of the Pakistan.

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Material and methods

The experimental site is located in the Department of Botany at the Karachi University Campus and seedling growth experiment was carried out in pots in green house. 1% solution of red chilli or coriander powder prepared by weighing one g of spice powder then dissolve in 99 ml of distilled water to make up the volume up to 100 ml and subsequent concentrations 1%, 2%, 3%, 4% and 5% were prepared respectively. The fruit of red chilli and seeds of coriander powder was kept in boiling so that convert into solution completely. The certified seeds of cowpea and mung bean were purchased from market and imbibed in water for an hour to break seed dormancy. The beans seeds surface was sterilized with 1N bleach solution for three minutes and rinsed by distilled water to remove any type of fungal contamination. The plastic pots were 7.3 cm in diameter and 9.6 cm in depth and were filled with ratio of one-part manure and three parts garden loam. Dig 1 cm holes of soil from above the surface of pots, at least 5 holes and buried the 2-2 seeds of one type of plant into pot. The pots were water daily and under the influence of sunlight, seeds were able to germinate uniformly in two weeks. One seedling was grown in each pot and ten ml of powder extract of *Capsicum annuum* L. and *Coriandrum sativum* L. were provided into the respective pots. The experiment was completely randomized. The pots were reshuffled weekly to avoid light, shade or any other climatic factors. There were five replicates of each treatment. The seedlings were uprooted from the pots after five weeks and washed with tap water. The root, shoot and seedling length and numbers of leaves were recorded. The removed seedlings were kept in oven at 80 °C for 24 hours for the determination of dry weight. Root, leaf, shoot and total plant fresh and dry weight were recorded. Root / shoot ratio, leaf weight, specific leaf area and leaf area ratio were determined according to REHMAN & IQBAL (2009).

The obtained data was statistically analyzed by ANOVA and DMRT (Duncan Multiple Range Test) ($p < 0.05$) using software packages SPSS version 14.0 on personal computer.

Results

The treatment of different concentrations of red chilli and coriander aqueous powder extract showed variable effects on growth performance of cowpea and mung bean (Table 1-4). Red chilli extract treatment at 4% significantly ($p<0.05$) decreased root, shoot, seedling height, leaf area and total fresh weight of cow pea. The significant decline in leaf weight ratio of cow pea was found at 3% red chilli extract treatment. The treatment of red chilli powder extract at 3% was brought a significant decrease in shoot growth, number of leaves and leaves dry weight of mung bean. The treatment of red chilli at all concentration showed nonsignificant effect on root, shoot dry weight and specific leaf area of cow pea, whereas, root fresh weight and root shoot ratio of mung bean. Shoot, root, seedling length, number of leaves and leaf size of cow pea was highly decreased at 5% coriander aqueous powder extract treatment. Coriander extract treatment at 1% treatment produced significantly lower number of leaves in mung bean. The coriander extract treatment at 5% significantly affected root, shoot and total plant dry weight of mung bean. Leaf weight ratio, of mung bean greatly affected by 5% of coriander powder extract treatment.

Table 1. Growth of cowpea in various concentrations of red chilli powder extract.

Seedling growth characteristic	Treatments aqueous powder extract concentration (%)					
	0	1	2	3	4	5
Shoot length (cm)	69.31 f ± 0.070	68.86 e ± 0.017	66.67 d ± 0.017	66.56 c ± 0.015	60.32 b ±0.006	60.00 a ±0.001
Root length (cm)	12.75 f ± 0.020	12.05 e ± 0.020	10.80 d ± 0.011	9.87 c ± 0.004	9.50 b ±0.040	8.27 a ±0.016
Seedling length (cm)	82.05 f ± 0.054	80.91 e ± 0.006	77.47 d ± 0.099	76.43 c ± 0.018	69.82 b ± 0.044	68.27 a ± 0.009
Number of leaves	15.00 ae ± 0.00	13.66 d ± 0.710	12.33 c ± 0.062	12.00 bc ± 0.001	11.00 ab ± 0.010	10.33 a ± 0.023
Leaf area (cm^2)	48.65 d ± 0.037	42.91 d ± 0.007	38.00 c ±0.408	35.33 b ±0.082	35.58 b ± 0.129	30.85 a ± 0.042
Root fresh weight (g)	0.880 d ± 0.020	0.860 d ± 0.006	0.800 c ± 0.004	0.640 b ± 0.004	0.620 b ± 0.018	0.440 a ± 0.008
Shoot fresh weight (g)	1.72 e ± 0.006	1.42 d ± 0.006	1.34 c ± 0.004	1.33 c ± 0.004	1.02 b ± 0.020	0.850 a ± 0.012
Leaves fresh weight (g)	1.15 d ± 0.009	1.01 ± 0.230	0.870 bc ± 0.006	0.640 ab ± 0.012	0.620 ab ± 0.005	0.440a ± 0.008
Total plant fresh weight (g)	3.30 d ± 0.106	2.290 c ± 0.206	2.350 c ± 0.16	2.11 bc ± 0.012	1.890 ab ± 0.092	1.760 ab ± 0.082
Root dry weight (g)	0.180 a ± 0.430	0.190 a ± 0.025	0.310 a ± 0.004	0.170 a ± 0.004	0.120 a ± 0.005	0.100 a ± 0.198
Shoot dry weight (g)	0.850 e ± 0.012	0.720 d ± 0.004	0.660 c ± 0.013	0.680 c ± 0.017	0.520 b ± 0.004	0.310 a ± 0.004
Leaves dry weight (g)	0.280 a ± 0.008	0.260 a ± 0.006	0.250 a ± 0.011	0.240 a ± 0.008	0.185 a ± 0.005	0.100 a ± 0.220
Total plant dry weight (g)	2.180 b ± 0.428	2.020 ab ± 0.220	1.510 a ± 0.070	1.210 a ± 0.039	0.980 a ± 0.041	0.780 a ± 0.020
Root / Shoot ratio	0.297 b ± 0.037	0246 a ± 0.032	0.350 a ± 0.002	0.310 a ± 0.036	0.316 ab ± 0.032	0.320 a ± 0.063
Leaf weight ratio	0.450 ab ± 0.007	0.280 ab ± 0.015	0.220 a ± 0.004	0.230 a ± 0.006	0.310 b ± 0.011	0.340 b ± 0.005
Specific leaf area ($cm^2\ g^{-1}$)	23.260 a ± 0.004	38.930 a ± 0.122	43.270 a ± 0.148	53.190 a ± 0.169	37.540 a ± 0.104	47.160 a ± 0.242
Leaf area ratio ($cm^2\ g^{-1}$)	10.450 c ± 0.107	10.990 c ± 0.330	9.910 a ± 0.042	12.620 b ± 0.036	11.800 d ± 0.057	11.550 e ± 0.102

Number followed by the same letters on the same column are not significantly different ($p<0.05$) according to Duncan's Multiple Range Test. Symbol used: ± = Standard error.

Table 2. Growth of mung bean in various concentrations of red chilli powder extract.

Seedling growth characteristic	Treatments aqueous powder extract concentration (%)					
	0	1	2	3	4	5
Shoot length (cm)	48.210 a ± 0.048	44.130 e ± 0.041	43.00 d ± 0.025	42.810 c ± 0.020	42.330 b ±0.213	40.230 b ±0.052
Root length (cm)	12.43 a ± 0.094	10.96 e ± 0.251	11.370 c ± 0.094	11.000 d ± 0.001	8.060 d ±0.043	6.900 b ±0.231
Seedling length (cm)	60.640 b ± 0.140	55.090 a ± 0.029	54.370 b ± 0.071	53.810 b ±11.767	50.390 b ± 0.172	47.130 b ± 0.273
Number of leaves	10.00 a ± 0.001	8.660 c ± 0.249	8.000 b ± 0.002	7.870 ab ± 0.462	7.510 a ± 0.427	7.120 a ± 0.241
Leaf area (cm^2)	22.800 a ± 0.218	21.450 e ± 0.114	20.450 d ±0.085	20.300 c ±0.091	18.000 c ± 0.001	17.000 b ± 0.002
Root fresh weight (g)	0.390 a ± 0.009	0.360 a ± 0.004	0.230 a ± 0.013	0.210 a ± 0.006	0.100 a ± 0.223	0.090 a ± 0.004
Shoot fresh weight (g)	1.630 a ± 0.165	1.410 e ± 0.006	1.400 d ± 0.005	1.210 d ± 0.013	1.150 c ± 0.004	1.010 b ± 0.006
Leaves fresh weight (g)	1.280 b ± 0.021	0.520 e ± 0.006	0.720 a ± 0.002	0.690 d ± 0.004	0.640 c ± 0.004	0.620 b ± 0.005
Total plant fresh weight (g)	3.300 a ± 0.019	2.290 c ± 0.011	2.350 b ± 0.11	2.110 b ± 0.017	1.890 b ± 0.229	1.720ab ± 0.006
Root dry weight (g)	0.170 a ± 0.004	0.150 bc ± 0.004	0.190 b ± 0.004	0.160 c ± 0.004	0.040 b ± 0.006	0.020 a ± 0.017
Shoot dry weight (g)	1.030 a ± 0.004	1.010 e ± 0.006	0.640 f ± 0.004	0.410 d ± 0.004	0.350 c ± 0.008	0.210 b ± 0.002
Leaves dry weight (g)	0.980 a ± 0.004	0.860 f ± 0.004	0.680 e ± 0.004	0.590 c ± 0.008	0.550 b ± 0.004	0.780 b ± 0.220
Total plant dry weight (g)	2.180 b ± 0.428	2.020 ab ± 0.220	1.510 a ± 0.070	1.210 a ± 0.039	0.980 a ± 0.041	0.780 a ± 0.020
Root / Shoot ratio	0.297 a ± 0.004	0.291 a ± 0.043	0.304 a ± 0.002	0.296 a ± 0.020	0.216 a ± 0.028	0.192 a ± 0.006
Leaf weight ratio	0.450 a ± 0.001	0.425 a ± 0.011	0.450 a ± 0.001	0.450 ab ± 0.004	0.602 a ± 0.051	0.705 b ± 0.014
Specific leaf area ($cm^2\ g^{-1}$)	23.260 a ± 0.242	24.900 b ± 0.095	30.070 c ± 0.052	31.710 d ± 0.100	30.500 c ± 0.097	30.900 c ± 0.176
Leaf area ratio ($cm^2\ g^{-1}$)	10.450 a ± 0.069	10.610 a ± 0.112	13.540 b ± 0.070	16.770 c ± 0.155	18.360 d ± 0.184	21.790 e ± 0.154

Number followed by the same letters on the same column are not significantly different ($p<0.05$) according to Duncan's Multiple Range Test. Symbol used: ± = Standard error.

Table 3. Growth of cowpea in various concentrations of coriander powder extract.

Seedling growth characteristic	Treatments aqueous powder extract concentration (%)					
	0	1	2	3	4	5
Shoot length (cm)	69.30 f ± 0.040	65.87 d ± 0.067	64.56 c ± 0.157	68.92 e ± 0.195	62.18 b ±0.008	57.00 a ±0.278
Root length (cm)	12.75 c ± 0.016	10.80 b ± 0.040	15.05 e ± 0.012	12.80 c ± 0.173	13.65 d ±0.167	8.60 a ±0.040
Seedling length (cm)	82.05 f ± 0.022	76.67 c ± 0.154	79.61d ± 0.030	81.72 e ± 0.150	75.83 b ± 0.011	65.60 a ± 0.086
Number of leaves	15.00 e ± 0.00	10.30 b ± 0.230	10.23 b ± 0.020	12.66 d ± 0.230	11.00 c ± 0.010	9.00 a ± 0.001
Leaf area (cm^2)	48.65 d ± 0.037	40.38 c ± 0.033	46.00 d ±0.040	40.41 c ±0.004	30.33 b ± 0.008	22.16 a ± 2.736
Root fresh weight (g)	0.88 b ± 0.004	0.930 c ± 0.004	0.820 a ± 0.004	1.00 d ± 0.002	0.950 e ± 0.004	0.810 a ± 0.004
Shoot fresh weight (g)	1.72 d ± 0.013	1.73 d ± 0.008	1.44 c ± 0.008	1.41 c ± 0.004	1.28 b ± 0.020	1.140 a ± 0.008
Leaves fresh weight (g)	1.15 d ± 0.009	0.970 ab ± 0.212	0.950 bc ± 0.008	0.850 ab ± 0.144	0.720 ab ± 0.004	0.610 a ± 0.008
Total plant fresh weight (g)	3.30 c ± 0.115	3.39 bc ± 0.206	3.70 bc ± 0.122	3.30 bc ± 0.017	3.53 b ± 0.149	2.96 a ± 0.038
Root dry weight (g)	0.220 a ± 0.400	0.210 a ± 0.004	0.300 a ± 0.005	0.240 a ± 0.008	0.200 a ± 0.004	0.180 a ± 0.010
Shoot dry weight (g)	0.210 d ± 0.012	0.130 b ± 0.007	0.200 d ± 0.018	0.150 a ± 0.015	0.130 c ± 0.008	0.160 a ± 0.004
Root / Shoot ratio	0.180 a ± 0.005	0.230 c ± 0.001	0.220 bc ± 0.003	0.200 ab ± 0.009	0.300 d ± 0.010	0.270 c ± 0.008
Leaf weight ratio	0.850 d ± 0.004	0.880 d ± 0.004	0.740 c ± 0.005	0.610 b ± 0.012	0.800d d ± 0.034	0.590 a ± 0.007
Specific leaf area ($cm^2\ g^{-1}$)	0.280 d ± 0.002	0.170 b ± 0.002	0.250 c ± 0.010	0.180 b ± 0.004	0.170 ab ± 0.002	0.170 a ± 0.004
Leaf area ratio ($cm^2\ g^{-1}$)	2.180 c ± 0.007	2.30 c ± 0.244	2.400 c ± 0.020	1.98 b ± 0.027	1.94 b ± 0.023	1.510 a ± 0.012

Number followed by the same letters on the same column are not significantly different ($p<0.05$) according to Duncan's Multiple Range Test. Symbol used: ± = Standard error.

Table 4. Growth of mung bean in various concentrations of coriander powder extract.

Seedling growth characteristic	Treatments aqueous powder extract concentration (%)					
	0	1	2	3	4	5
Shoot length (cm)	48.21 e ± 0.008	45.31 b ± 0.004	45.42 c ± 0.029	46.13 d ± 0.010	46.22 d ±0.027	40.23 a ±0.016
Root length (cm)	12.43 d ± 0.020	13.37 de ± 0.010	11.90 a ± 0.040	12.30 c ± 0.062	12.33 c ±0.009	6.90 b ±0.016
Seedling length (cm)	60.64 e ± 0.014	56.68 d ± 0.014	57.32 b ± 0.056	58.43 c ± 0.057	58.55 c ± 0.030	47.13 a ± 0.029
Number of leaves	10.00 b ± 0.00	8.35 a ± 0.781	9.00 b ± 0.00	10.12 b ± 0.409	9.66 b ± 0.034	7.22 b ± 0.001
Leaf area (cm^2)	22.80 a ± 0.080	22.97 ab ± 0.040	23.80 b ±0.080	25.00 c ±0.062	22.90 a ± 0.022	17.00 c ± 0.81

Root fresh weight (g)	0.390 a ± 0.007	0.650 b ± 0.010	0.980 d ± 0.047	0.610 b ± 0.004	0.780 c ± 0.006	0.090 b ± 0.004
Shoot fresh weight (g)	1.630 c ± 0.004	1.650 c ± 0.004	1.770 d ± 0.017	1.720 e ± 0.006	1.540 b ± 0.004	1.010 a ± 0.011
Leaves fresh weight (g)	1.280 f ± 0.004	1.090 d ± 0.010	1.090 d ± 0.010	0.950 a ± 0.019	1.210 e ± 0.004	0.620 c ± 0.006
Total plant fresh weight (g)	3.30 b ± 0.125	3.390 c ± 0.017	3.700 e ± 0.048	3.300 b ± 0.008	3.530 d ± 0.004	1.720 a ± 0.006
Root dry weight (g)	0.170 b ± 0.005	0.250 d ± 0.004	0.480 e ± 0.008	0.210 c ± 0.004	0.190 b ± 0.004	0.070 a ± 0.008
Shoot dry weight (g)	1.030 b ± 0.004	1.250 d ± 0.004	1.370 f ± 0.004	1.300 e ± 0.004	1.140 c ± 0.004	0.910 a ± 0.004
Leaves dry weight (g)	0.980 a ± 0.217	0.590 a ± 0.008	0.550 a ± 0.097	0.470 a ± 0.004	0.610 a ± 0.005	0.530 ab ± 0.019
Total plant dry weight (g)	2.180 b ± 0.210	2.090 b ± 0.010	2.400 c ± 0.012	1.980 b ± 0.010	1.940 b ± 0.008	1.510 a ± 0.021
Leaf weight ratio	0.450 e ± 0.007	0.280 c ± 0.150	0.220 c ± 0.004	0.230 b ± 0.006	0.310 d ± 0.001	0.340 d ± 0.005
Specific leaf area ($cm^2\ g^{-1}$)	23.260 a ± 0.094	38.390 c ± 0.123	43.270 d ± 0.146	53.190 f ± 0.159	37.540 b ± 0.104	47.160 e ± 0.242
Leaf area ratio ($cm^2\ g^{-1}$)	10.450 b ± 0.107	10.990 c ± 0.033	9.910 a ± 0.042	12.620 d ± 0.036	11.800 d ± 0.067	16.550 e ± 0.103

Number followed by the same letters on the same column are not significantly different ($p<0.05$) according to Duncan's Multiple Range Test. Symbol used: ± = Standard error.

The seedlings of cowpea were tested to the tolerance to different concentrations of red chilli and coriander powder extract. Red chili extract treatment at 1, 2, 3, 4 and 5% gradually decreased tolerance indices by 94.50, 84.70, 77.41, 74.50 and 65.79% in seedlings of cowpea. The treatment of coriander extract treatment at 5% showed the lowest tolerance (67.45%) indices. However, on comparison, the seedlings of cowpea showed more tolerance to coriander powder extract than red chili extract at 5%. Red chilli extract treatment at 4 and 5% showed tolerance indices by 64.84 and 55.51% in seedlings of mung bean. Coriander extract treatment at all concentrations showed better tolerance in seedlings of mung bean as compared to red chilli extract at similar concentrations.

Discussion

The studies on interactions and competition for resources among plants was carried out and suggested for the use in modifying the pattern of crop cultivation and for the increase in yields [MAJEED & al. 2017]. The treatment of red chili and coriander aqueous powder extract influenced on seedling growth performances of cowpea and mung bean. The agrochemical groups are naturally occurred in plant [KUTI & al. 1990] and produced favorable and toxic impact on the growth of surrounding plants. It was found that the release of chemical compounds from both home spices in the given substrate affected growth performance of both beans. The different response in seedling growth parameters of bean seems attributable to the level of red chili and coriander powder extract treatments. Allelopathic compounds from plants residues called allelochemicals and may work as inhibitor or beneficial for growth of subsequent plants [RICE, 1984]. The maximum

inhibitory allelopathic effect of bindweed (*Convolvulus arvensis* L.) vegetative part at 10% on seed vigor index for millet and basil plants was observed [FATEH & al. 2012]. The inhibitory substances from *C. annuum* and *C. sativum* released in the soil which apparently showed allelopathic potential and might be cause for significant decline in growth characteristics of cowpea and agreed with the findings of ELLS & MCSAY (1991) on cucumber seedlings by alfalfa plant residues. Capsaicin have a powerful allelopathic effect on growth of plant [CHO & al. 1992] and growth of weed [GONZALEZ & al. 1997]. The treatment of both spices at 2% level further decreased root and shoot growth of cowpea. MOOSAVI & al. (2011) also found significantly decreased in shoot and root growth of *Vigna radiata* L. with aqueous extract of leaf, stem and root of sorghum. The treatment of ccoriander powder extract showed beneficial and harmful effects on the seedling characteristics of cowpea. The low concentration of coriander powder at 1% slightly increased and a higher concentration decreased the seedling dry weight of cowpea. The presence of phenols and tannins from *Jatropha curcas* (5-20%) as allelochemicals showed inhibitory effect on green chilli and stimulatory on sesame [REJILA & VIJAYAKUMAR, 2011].

Conclusions

It was concluded that aqueous powder extract of red chili and coriander at 5% influenced on seedling growth and tolerance index of cowpea and mung bean. The availability of toxic allelochemicals compounds from both spices in substrate showed strong allelopathic potential activity for seedling of cowpea and mung bean.

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PHARMACEUTICALLY ACTIVE CELL BIOMASS GROWTH PATTERN UNDER CELL SUSPENSION CULTURE OF *COMMIPHORA WIGHTII* – A CRITICALLY ENDANGERED MEDICINAL PLANT

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Abstract: One of the medicinal treasures of Indian Ayurveda is *Commiphora wightii* which is being used for treating many diseases due to the presence of an oleo-gum-resin (guggul gum) that is having a number of secondary metabolites which are bioactive principles for a number of medicinally important ayurvedic preparations. Over-exploitation of the plant for this resin led it to the verge of extinction and need to develop an alternative way to produce this guggul gum has become imperative. The present study was aimed to understand the behaviour and growth pattern of cell suspension culture of *C. wightii*, which can foster the way to produce secondary metabolites from *in-vitro* cultures or secondary metabolite rich cell biomass. For this, callus was initiated from immature embryos collected from seed raised mature plants and tissue culture raised mature plants on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cell suspension culture was established for both the samples in Gamborg's B5 medium with 0.5 mg/l 2,4-D and hormone free medium. Study showed a comparable growth where good growth was observed in medium containing hormone than medium without hormone. In tissue culture raised plants highest biomass was observed on 27th day which is 17.3149 ± 0.71 gms in hormone supplemented medium while in hormone free medium highest biomass was obtained on 27th day which is 14.6219 ± 1.28 gms. Whereas in seed derived plants highest biomass produced in medium containing hormone was on 27th day that is 14.9060 ± 0.73 gms while in hormone free medium highest biomass was obtained on 27th day that is 11.2113 ± 0.74 gms.

Keywords: cell biomass, *Commiphora wightii*, fresh weight, growth kinetics, suspension culture.

Introduction

Commiphora wightii (Arn.) Bhandari (Family Burseraceae) is an important and pharmaceutically valuable medicinal plant of arid and semi-arid regions of the Indian sub – continent [URIZAR & MOORE, 2003; DENG, 2007]. It is commonly known as 'Guggul' due to the presence of an oleo-gum-resin which is composed mainly of alcohols, steroids, diterpenes and sterols [VERMA & al. 1998] and the main active constituents are guggulsterone E and Z [EL ASHRY & al. 2003]. Many biochemical studies have been done on chemical composition of this exudate showing presence of guggulsterone I, II, III, V, Z, E and ctadecan-1,2,3,4-tetrol [PATIL & al. 1972, 1973], Identified guggulsterone E & Z, guggulsterone-I, myrrhanol-A and myrrhanone-A, guggulsterone-M, dihydro guggulsterone-M, guggulsterol-Y [MESELHY, 2003], Presence of essential oils, myrecene, dimycerene and Polymyrerecene [JAIN & GUPTA, 2006], Presence of Quinic acid, Citric acid myo-inositol and Glycin in leaves, stem and resin through metabolite profiling [BHATIA & al. 2018].

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Due to the presence of these diverse groups of compound *C. wightii* is used for therapeutic purpose. It is used in treating rheumatoidism and arteriosclerosis [GUJRAL & al. 1960; SATYAVATI & al. 1969]. The active constituents of this gum are used for treatment of hyper cholesterolemia, ulcers, obesity etc. [SATYAVATI, 1990], for the treatment of neurological disorders, hypertension and asthma [MAHESHWARI, 2010] and to prevent the progression of cancerous cells [SHISHODIA & al. 2007]. Antioxidant and cytotoxic activity from ethyl acetate extract of *C. wightii* under *in-vitro* condition has also been reported [ZHU & al. 2001]. It has also identified as weight loss agent [KIMURA & al. 2001] and cholesterol lowering agent [JAIN & GUPTA, 2006].

The natural population of this plant is declining because of relentless harvesting of resin from wild plants through tapping by local people for economic benefits. Other reasons of its declining population are slow growth and poor germination rate [YADAV & al. 1999] due to which it has been listed as critically endangered species by IUCN [VED & al. 2015, e.T31231A50131117]. Other than conventional breeding, biotechnological aspects have been explored by many researchers to conserve this valuable plant. Micropropagation has been achieved for *C. wightii* using different explants like nodal segments [BARVE & MEHTA, 1993; SONI, 2010; PARMAR & KANT, 2012], shoot tips, nodes, internodes and leaves [SINGH & al. 2010], seedling explants [YUSUF & al. 1999; KANT & al. 2010] and apical and axillary meristem [BHARDWAJ & ALIA, 2019]. Somatic embryogenesis in *C. wightii* was achieved by repetitive reciprocal transfer of callus between MS basal medium and MS supplemented with plant growth regulators [KUMAR & al. 2003]. Studies on *in vitro* guggulsterone production have been done for the production of these compounds without destructing the natural population of *C. wightii*. Production of guggulsterone in callus culture was reported that has been induced from different explants like leaves, zygotic embryos and stem [MATHUR & al. 2007a]. Guggulsterone production has also been reported in shake flask and bioreactors [MATHUR & al. 2007b]. Enhanced guggulsterone production in cultures was observed in the presence of Morphactin and 2iP [TANWAR & al. 2007], Mesquite Gum and Gum Arabic [DASS & RAMAWAT, 2009] and growth retardants with fungal elicitors [SUTHAR & RAMAWAT, 2010].

Production of secondary metabolites in suspension culture is also affected by the growth of cells in liquid medium and therefore it is necessary to understand the growth pattern of cells in culture conditions. Growth kinetics of cells in liquid medium has been studied on many plant species through Packed Cell Volume in *Phoenix dactylifera* L. suspension culture [AL-KHAYRI, 2012], Fresh weight and cell viability in *Scrophularia striata* Boiss. suspension culture [ARDESTANI & al. 2015] and Settled Cell Volume in *Sorghum bicolor* suspension culture [RAMULIFHO & al. 2019]. So, the present study was conducted to analyse the growth pattern of *C. wightii* cell suspension culture which can pave the path for obtaining healthy cell biomass and for enhancement of guggulsterone production.

Materials and methods

Plant material

Immature fruits were collected from healthy seed-derived plants of *C. wightii* growing at AFRI nursery and from tissue culture raised plants growing at AFRI TC field trial, Jodhpur (grown *in vitro* as in KANT & al. 2010).

Explant preparation

Immature fruits were soaked in water. The floating fruits were discarded. Only the settled fruits were used as explant source. These were washed with tween 20 followed by bavestein and streptomycin (Himedia) treatment. Finally fruits were surface sterilized with 5% NaOCl (Sigma-Aldrich). Embryos were scooped out from immature fruits for inoculation.

Callus induction and establishment

Embroys were inoculated on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D (Sigma-Aldrich) as reported best concentration for callus induction earlier by our team [PARMAR & KANT, 2014]. Establishment of callus was done for both the source mother plants separately by regular sub-culturing after every 4 week on same medium. Further, callus was inoculated on semi solid Gamborg's B5 medium without any hormone for induction of embryogenesis [PARMAR & KANT, 2014].

Establishment of cell suspension cultures

C. wightii suspension cultures were initiated by inoculating non embryogenic callus (~1 g per flask) in 100-ml Erlenmeyer flasks containing 30 ml liquid medium. The medium consist of same basal salt concentrations of Gamborg's B5 medium which was used for callus induction but without agar. The cultures were initiated for both the source plants in Gamborg's B5 medium Supplemented with 0.5 mg/l 2,4-D and Gamborg's B5 medium without any hormone. The suspension cultures were incubated on a rotatory shaker (Adolf Kuhner AG LSX SMX 1200) at 120 rpm under 16-hr photoperiod in white fluorescent light and at 25 ± 2 °C.

Suspension growth pattern analysis

Cell suspension Culture's growth pattern analysis was done by fresh weight and dry weight methods. Cells were collected from flasks every 3rd day after inoculation on a pre-weighed Whatmann filter paper disc by filtering the suspension culture. Total weight of the filter paper and cells were determined and then weight of the filter paper was subtracted to obtain the fresh weight (FW) of cells. Filter paper containing the cells was placed in an oven at 60 °C for 24 hrs and weighed at regular intervals until the weight remains constant. Dry weight (DW) was obtained by subtracting weight of paper with dried cells from pre-weighed paper's weight.

Determination of cell viability

Viability of cells in suspension culture was checked by Evan's blue staining method. Cells were taken from suspension culture every 3rd day after inoculation and stained with 0.4% Evan's blue dye (Himedia). Stained cells were observed under microscope (Nikon Optiphot - 2) at 20x magnification.

Results

Establishment of callus culture

Callus was initiated from immature embryos for both the source plants and per cent callusing was recorded (Table 1). Tissue culture raised plant's explant showed a little higher percentage than seed derived plant's explant. The non – embryogenic callus converted to embryogenic callus when cultured on Gamborg's B5 medium without any hormone (Figure 1). This showed the progressive growth of embryogenic callus on semi solid medium which can also be induced and established in suspension culture for production for guggulsterones.

Table 1. Initiation of callus on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D.

S.No.	Mother plant	% Callusing	Callus colour and texture
1	Tissue culture raised plants	66.66	Pinkish white, fragile
2	Seed derived plants	58.33	Pinkish white and light brown, fragile

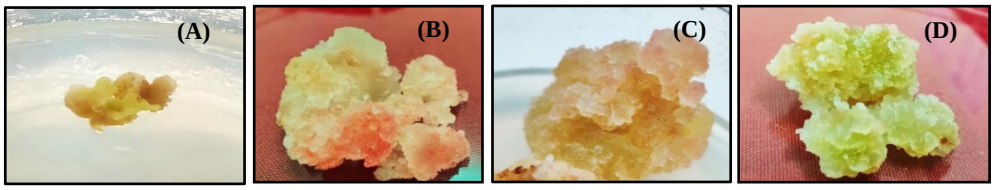

Figure 1. Conversion of non - embryogenic callus into embryogenic callus. (A) Induction of callus on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D. (B) Multiplication of callus on same medium. (C) Non-embryogenic callus converting into embryogenic callus on Gamborg's B5 medium without any hormone. (D) Embryogenic callus.

Establishment of cell suspension culture

Cultures initiated from tissue culture raised plant's explant showed comparatively good growth in medium containing hormone 2,4-D (Figure 2) as compared to cultures in medium without hormone (Figure 3) because 2,4-D is responsible for higher rate of cell division. Similar pattern was observed in cultures initiated from seed derived plant's explant (Figures 4, 5). While comparing the two mother plants, tissue culture raised plants gave better response as compare to seed derived plants in terms of having dense biomass which is clearly seen in the flasks.

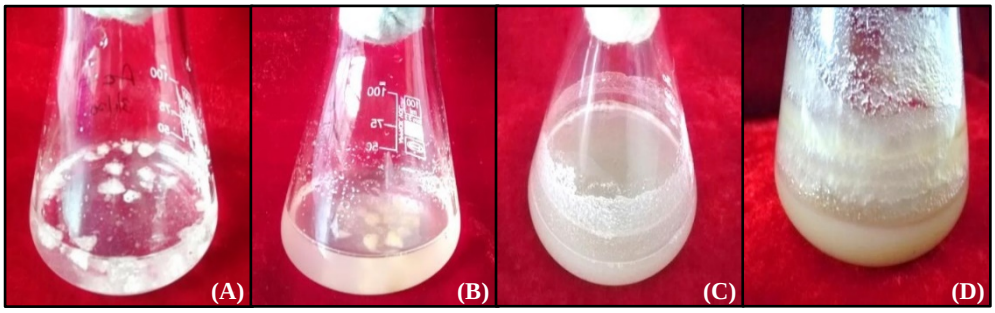

Figure 2. Tissue culture raised plants: Growth pattern of culture suspension in shake flask containing medium with hormone 2,4-D (A-D) A. 1st Day, B. 10th Day, C. 20th Day, D. 30th Day.

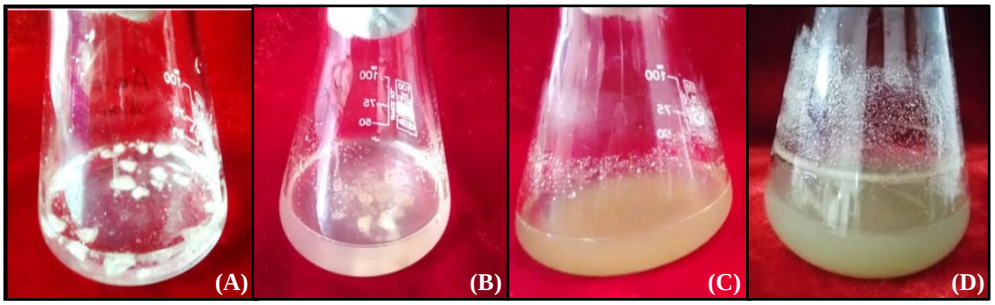

Figure 3. Tissue culture raised plants: Growth pattern of suspension culture in shake flask containing medium without any hormone (A-D) A. 1st day, B. 10th day, C. 20th day, D. 30th day.

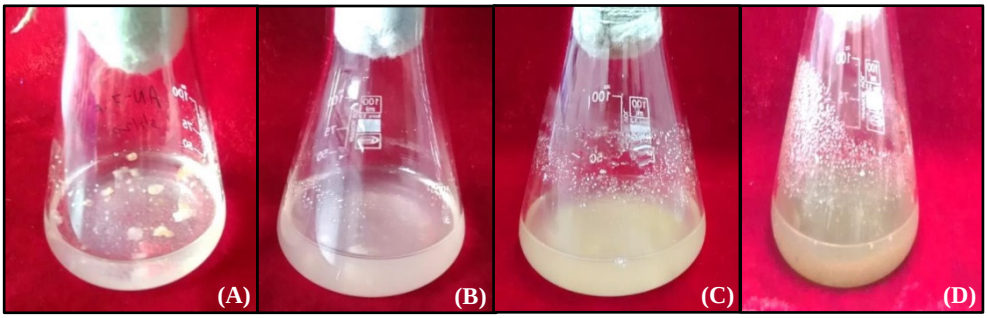

Figure 4. Seed derived plants: Growth pattern of suspension culture in shake flask containing medium with hormone (A-D) A. 1st day, B. 10th day, C. 20th day, D. 30th day.

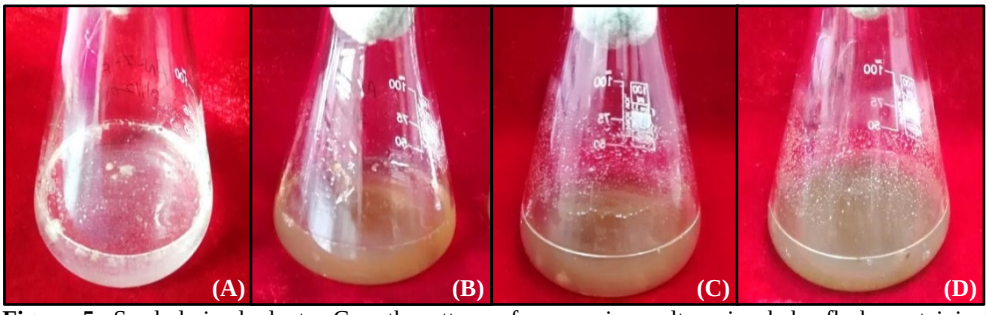

Figure 5. Seed derived plants: Growth pattern of suspension culture in shake flask containing medium without any hormone (A-D) A. 1st day, B. 10th day, C. 20th day, D. 30th day.

Growth pattern analysis of suspension culture

Tissue culture raised plants: Fresh weight analysis showed that growth of suspension culture in medium containing hormone grew with a short lag phase till day 9 that is the adaptive phase of culture to the new environment. This was followed with exponential growth till 21st day and after that stationary phase was achieved that finally culminated in the death phase marked by apoptosis due to nutrient exhaustion and cellular competition. Similarly suspension culture in medium without hormone showed quite similar growth pattern (Figure 6). Biomass produced was highest in medium containing hormone on 27th day which is 17.3149 ± 0.71 gms while in hormone free medium highest biomass was obtained on 27th day which is 14.6219 ± 1.28 gms. While comparing the dry weight, results was slightly different with highest dry mass on 18th day in cultures with hormone that is 0.4462 ± 0.00 gms and on 24th day in cultures without any hormone that is 0.4336 ± 0.01 gms. Growth pattern is shown for both the cultures in Figure 7.

Seed derived plants: Fresh weight analysis of these cultures exhibit lag phase till 12th day in medium containing hormone (2,4-D) and then grew exponentially till 27th day after that stationary phase was achieved leading to death phase. Similarly suspension culture in medium without hormone showed quite similar growth pattern with a gradual increase in growth with time (Figure 8). Biomass produced was highest in medium containing hormone on 27th day that is 14.9060 ± 0.73 gms while in hormone free medium highest biomass was obtained on 27th day that is 11.2113 ± 0.74 gms. Dry weight analysis

showed similar results as fresh weight method having highest dry mass on 27th day in medium with hormone that is 0.4325 ± 0.01 gms and in medium without hormone highest dry mass was obtained on 27th day that is 0.3899 ± 0.02 gms. Growth pattern is shown for both the cultures in Figure 9.

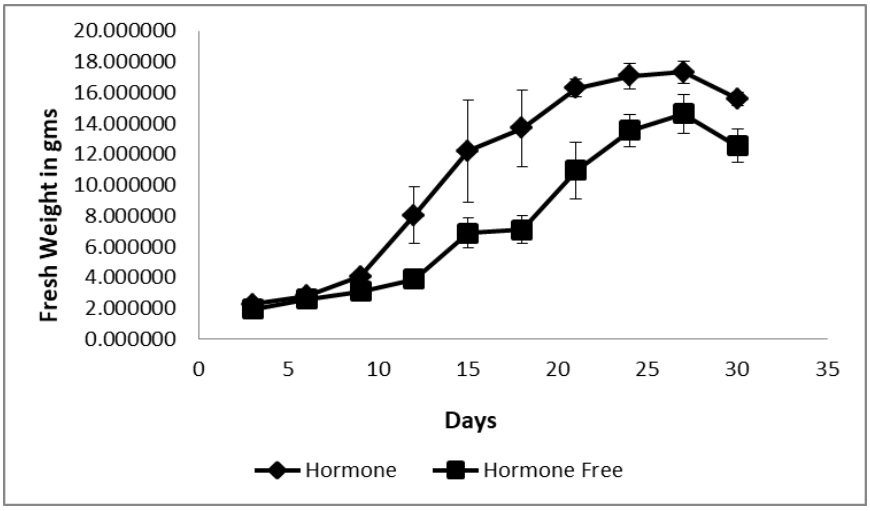

Figure 6. Growth pattern of culture initiated from tissue culture raised plant's explant in media containing hormone and without hormone by fresh weight method.

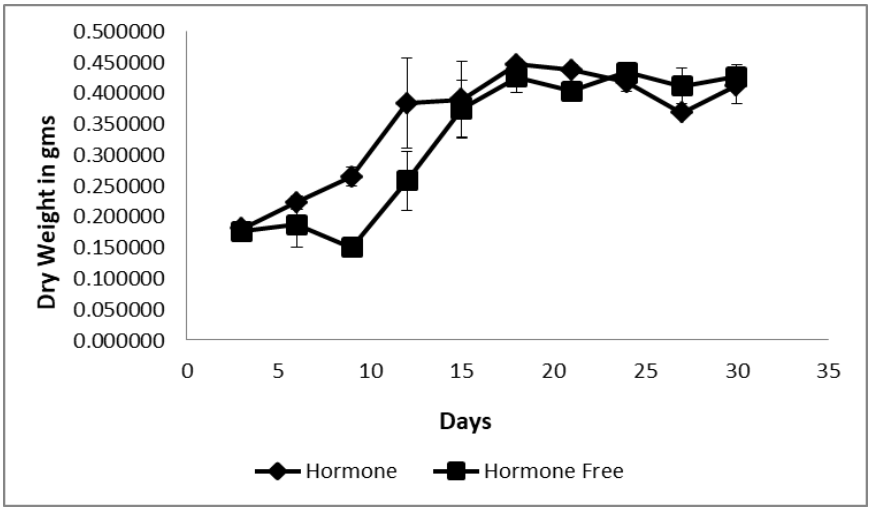

Figure 7. Growth pattern of culture initiated from tissue culture raised plant's explant in media containing hormone and without hormone by dry weight method.

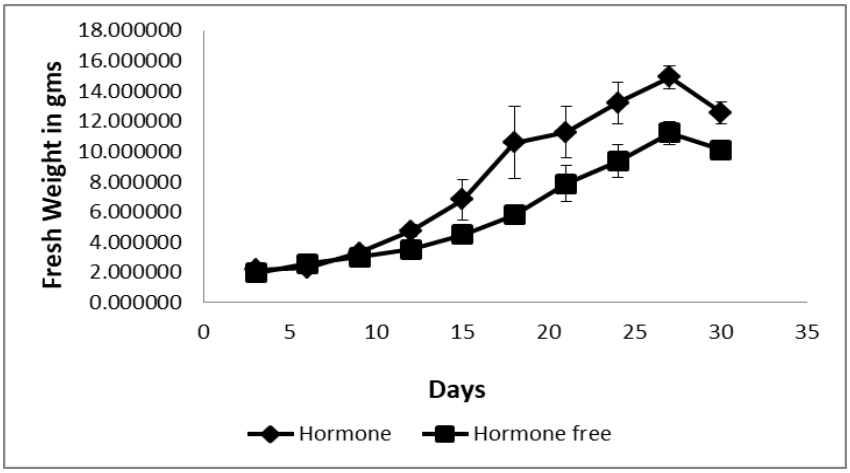

Figure 8. Growth pattern of culture initiated form seed derived plant's explant in media containing hormone and without hormone by fresh weight method.

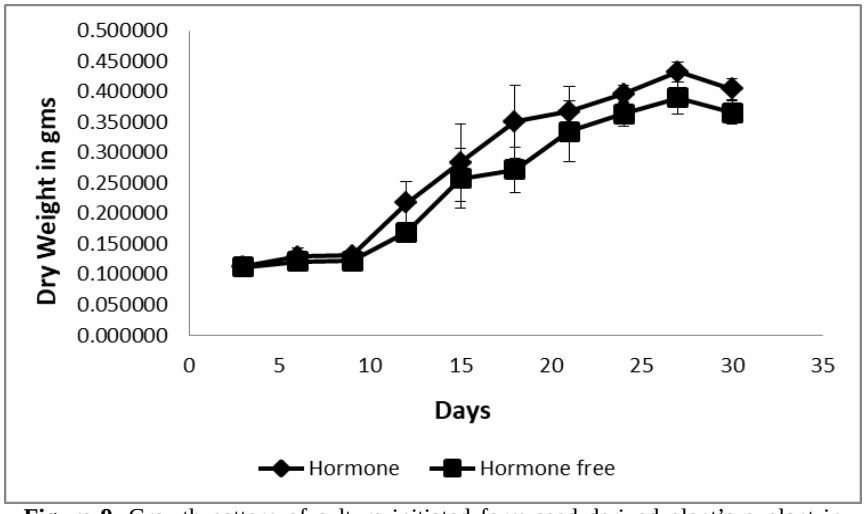

Figure 9. Growth pattern of culture initiated form seed derived plant's explant in media containing hormone and without hormone by dry weight method.

Determination of cell viability

Evan's blue staining revealed the viability of cells in suspension cultures of seed derived plant. In medium containing hormone a good growth was clearly seen in the culture along with more live cells in exponential phase which then decreased after the initiation of stationary phase (Figure 10). While in medium without any hormone showed slow growth along with less number of cells as compared to medium with hormone (Figure 11).

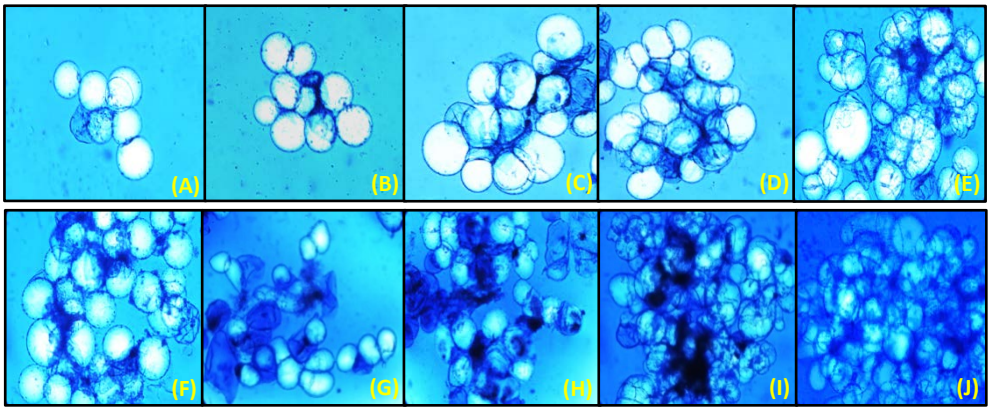

Figure 10. Evan's blue staining of suspension culture in Gamborg's B5 medium containing hormone, that are initiated from seed derived plant's callus showing live and dead cells with the time. (A) 3rd day (B) 6th day (C) 9th day (D) 12th day (E) 15th day (F) 18th day (G) 21st day (H) 24th day (I) 27th day (J) 30th day.

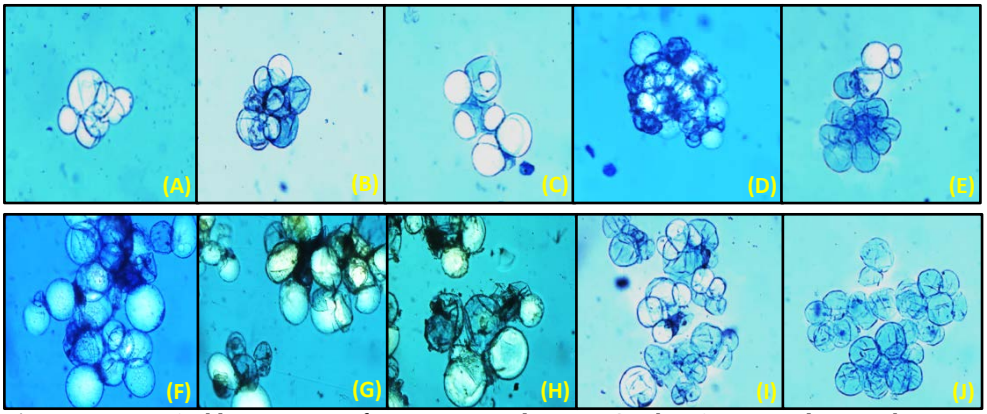

Figure 11. Evan's blue staining of suspension culture in Gamborg's B5 medium without any hormone, that are initiated from seed derived plant's callus showing live and dead cells by the time. (A) 3rd day (B) 6th day (C) 9th day (D) 12th day (E) 15th day (F) 18th day (G) 21st day (H) 24th day (I) 27th day (J) 30th day.

Discussion

In this study, we aimed to understand the growth pattern and behaviour of cells in suspension culture of *Commiphora wightii*. Plant cell growth in suspension culture can be determined and assessed by different methods like settled cell volume/packed cell volume, fresh weight and/or dry weight analysis, cell count/cell number [EVANS & al. 2003]. Here we have used fresh weight and dry weight method to make a growth curve to understand different phases of cell growth over a period of time. A typical growth curve is a sigmoidal curve having distinct phases like the lag phase (Adaptive phase), the exponential phase (Growth phase) and the stationary phase [GEORGE & al. 2008]. This study was done on two different mother plants and their growth was recorded in hormone (0.5 mg/l 2,4-D) and hormone free medium. In tissue culture raised plants suspension culture showed a lag phase

till 9th day followed with exponential phase till 21st day and then growth became steady with stationary phase in medium containing hormone. Whereas in medium without any hormone similar pattern has been seen but with less biomass produced. In seed raised plants suspension culture showed a different pattern with lag phase till 12th day followed by exponential phase till 27th day and then stationary phase was achieved in medium containing hormone. While in hormone free medium a gradual increase has been seen in growth of culture. After analysing the growth curve it can be suggested that sub - culturing of culture into fresh medium can be done in between 21st day to 27th day that is the end of exponential phase, which has been mentioned earlier in studies [STAFFORD & WARREN, 1991]. For maintaining a fine cell suspension culture, it is necessary to sub – culture at regular interval else the medium became exhaust or cells may produce toxic substances [BHOJWANI & RAZDAN, 1983]. In this study, it may be interesting to note that the growth of cell biomass in hormone free medium as quite comparable to that in case of hormone supplemented medium. KUMAR & al. (2004), reported the formation of resin canals which are the main source of guggulsterone synthesis in nature, at torpedo and cotyledonary stage of somatic embryos. So converting non embryogenic callus into differentiated embryogenic callus would have more amounts of guggulsterones. As we showed in figure 1, callus converted into embryogenic green callus when cultured on hormone free medium. Similarly culturing in hormone free suspension medium would also be useful in producing more amounts of guggulsterones *in vitro*. Thus going to hormone free medium is better when the targeted end product is a nutraceutical product.

Conclusions

The cell growth under suspension state is higher in hormone supplemented medium (0.5 mg/l 2,4-D in Gamborg's B5 medium) compared to the hormone free B5 medium. Evan's blue staining also indicates higher number of living cells in hormone supplemented medium when compared to hormone free medium. This can possibly be due to a faster cell division rate in presence of hormone (2,4-D). However, the cell growth was clearly observed even without 2,4-D in hormone free medium. It was also observed that the growth rate peaks early in hormone supplemented medium compared to hormone free medium in general. Interestingly it was also observed that tissue culture raised explant material was marginally better as culture starter compared to mother plants growing in nature. The study clearly indicates that cell biomass bulked up without any hormone under suspension state is a viable way to produce cell biomass for production of guggul nutraceuticals in future. Work on augmenting secondary metabolite production in these cells holds the key on which work is under progress.

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MOMORDICA CHARANTIA L. – NEW ACLIMATIZED PLANT IN ROMANIA. BOTANICAL CHARACTERS (REVIEW 1)

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Abstract: It has been performed an ample documentation about *Momordica charantia* L., a plant for the future, for food, nutrition and health security, respectively a plant with extended benefits in domains as: botanics, horticulture, phytochemistry, medicine, therapy health security a. o. This is the first published review on *M. charantia* in Romania in the frame of the project PN-II-PT-PCCA-2013-4-0995-160/2014 "Multifunctional and innovative products for safe and bioenhanced functional food from newly cultivated plants in Romania" developed at ICECHIM (INCDCP) Bucharest [ȘESAN, 2017; ȘESAN & al. 2018]. The present review contains the following aspects: introduction, botanical description, synonyms, common names, taxonomy, nomenclature, origin, distribution, biological actions. This review will be continued in next contributions about the same promising plant.

Key words: *Momordica charantia*, taxonomy, nomenclature, origin, distribution, biological actions.

Introduction

Momordica charantia L. is a medicinal and nutraceutical plant belonging to Cucurbitaceae family, widely grown in India, Asia, South America, Caraibbes, South Africa, used as food and in the traditional medicine (GROVER & YADAV, 2004; BELOIN & al. 2005; BEHERA & al. 2010; KUMAR & al. 2010; KUMAR & BHOWMIK, 2010; GUPTA & al. 2011; MAHMOUD & al. 2012, 2017; AGARWAL, 2014; NAGARANI & al. 2014a, b; ANILAKUMAR & al. 2015; UPADHYAY & al. 2015; TCHEGHEBE & al. 2016; DE OLIVEIRA & al. 2018; RAHMAN & al. 2018 a.o.), mentioned in different databases.

Botanical description

The most recent botanical description of *Momordica charantia* belongs to ASNA & al. (2020) in the most recent book of KOLE & al. (2020), published in Springer Verlag Nature Switzerland.

Classification. *Momordica charantia* belongs to the: Domain *Eukaryota*, Kingdom *Plantae*, Class *Dycotyledonnae*, Order *Cucurbitales*, *Cucurbitaceae* Family, *Momordica* genus, *M. charantia* L. species.

Synonoms known for *M. charantia* are: *M. chinensis* Spreng., *M. elegans* Salisb., *M. indica* L., *M. operculata* L., *M. sinensis* Spreng., *Sicyos fauriei* H. Lév. (https://uses.plantnet-project.otg/en/Momordica_charantia_(PROSEA).

Common Names: Bitter melon, Balsam pear, Bitter cucumber, Bitter pear, Karalla, Balsam apple, Cerasee, Carillacundeamor, Papailla, Melao de saoceatano, Bitter gourd,

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Sorosi, Karela, Kurela, Kor-kuey, Pava-aki, Salsamino, Sorossies, Pare, Peria, Karla, Margose, Goo-fah, Mara chean [RAHMAN & al. 2018].

After Pl@ntNet (http://publish.plantnet-project.org/project/riceweeds_en) and *Invasive Species Compendium* (ISC) (https://www.cabi.org/isc/), *Momordica charantia* L., is "a tropical and subtropical species belonging to the family Cucurbitaceae, widely grown for its edible fruit, which is among the most bitter of all fruits" (ISC). *M. charantia* is "an annual to perennial climbing, scandent or prostrate broadleaf herb growing up to 5 meters high; there is a central taproot, from the apex of which the stems spread to climb over any available support". **Stem** is herbaceous, tendril-bearing vine, being either hairless of slightly hairy. Green stems are well branched, slender, usually slightly 5-angled or ridged, and carry unbranched tendrils in the leaf axis (PLATE I). **Leaves** are up to 2-3 cm (ISC) up to 5 cm long (Pl@ntNet), with spiral tendrils at opposite sides; petioles 4-5 cm long, pubescent. The leaves are carried singly along the stems, and each leaf is 4-10(12) cm long, rounded in out line, and deeply (3)5-(7)9-lobed; the foliage has an unpleasant smell when crushed. **Flower** (PLATE II). Monoecious pale to deep yellow flowers with bract part way on peduncle, solitary in the upper leaf axils on 2-10 cm long stalks with a small leaf-like bract towards the base. Male flowers larger than female flowers and have a slender basal swelling which is continously with the base of sepale tube, which ends in five blunt sepals. There are five oval yellow petals 10-20 cm long, and five central stamens. Female flowers are similar to the male flowers but have a distinct warty swelling well below the base of the sepal tube and three stigmas. Male flowers appear first and usually exceed the number of female flowers by about 20:1. The flower opens at sunrise and remains open for one day. **Fruit** (PLATE II). The pendulous cylindrical fruits are egg-sharped and 2-10 cm long (up to 20 cm in cultivated varieties), and covered with longitudinal ridges and warts. At maturity, they turn orange to yellow, and the tips split into three and turn back to reveal the yellow pulp and the bright red arils that enclose the seeds which adhere to the inside of the fruit. Each of the flattened woody seeds is 5-9 mm long, and has finely pitted surfaces. The **seedlings** show epigeal germination, and resemble cucumber seedlings. The thick, brittle hypocotyls are 2-3 cm long and the first leaves shortly stalked, rounded, bluntly lobed and finely toothed.

Taxonomy and nomenclature

After SCHAEFER & RENNER (2010), genus *Momordica* comprises 47 species distributed in the warm tropics, chiefly in Africa, and with ~12 species in S-E Asia and Australia. All have unisexual flowers; from African species, 24 are dioecious, 23 monoecius, while all Asian species are dioecious.

Phylogenetic relationships in the order Cucurbitales and a new classification of the gourd family (Cucurbitaceae) has been presented by SCHAEFER & RENNER (2011a,b), as follows: Tribe 8. **Momordiceae** H. Schaef. & S.S. Renner, **stat**. **nov**., based on a diagnosis in Latin associated with Momordicinae M. Roem., Fam. Nat. Syn. Monogr. 2: 6. 1846 ('Momordiceae') – Typus: *Momordica* L. (Sp. Pl.: 1009. 1753). Thecae arcuate, duplicate or triplicate. Pollen large, 3-colporate, reticulate. Fruit small to large, fusiform or ovoid-ellipsoid or globose, usually spiny, tuberculate, winged or ridged, indehiscent or dehiscent by 3 valves or irregularly. Seeds few to several, yellow, brown or black, often with white, yellow or red arilli, medium-sized to large, subglobose to compressed; testa smooth or variously sculptured, margin often grooved. *n*=11 or 14 [BEEVY & KURIACHAN, 1996].

Genera and species: 60 species in 1 genus. – *Momordica* [60 spp., in tropical rainforest, deciduous forest and bushland, savannah and semi-deserts of tropical and subtropical Africa, Arabia, (sub)tropical Asia, Maleysia and Northeastern Australia; two species (*M. charantia*, *M. balsamina*), naturalized in Americas and Pacific islands]".

Maximum likelihood analyses performed by SCHAEFER & RENNER (2010) of 6257 aligned nucleotides of plastid, mitochondrial and nuclear DNA obtained for 122 accessions of *Momordica* and seven outgroups show that *Momordica* is monophylletic and consists of 11 well-supported clades. After the same authors, monoecy evolved from dioecy seven times independently, always in Africa and mostly in savanna species with low population densities. Leaky diocey, with occasional fruit-producing males, occurs in two African species and might be the first step in an evolutionary transition towards monoecy.

Dated biogeographic analyses suggest that *Momordica* originated in tropical Africa and the Asian species are the result of one long-distance dispersal event about 19 million years ago. The pantropical vegetable *M. charantia* is of African, not Asian origin as had previously been suggested [SCHAEFER & RENNER, 2010].

The Asiatic species of *Momordica* falls under three sects. Dioecious species like *M. cochinchinensis*, *M. dioica*, *M. sahyadrica*, *M. denticulata*, *M. denudata*, *M. clarkeana* and *M. subangulata* grouped under the sect. Cochinchinensis, and monoecious species *M. charantia* and *M. balsamina* under the sect. *Momordica* and *M. cymbalaria* under the sect. *Raphanocarpus* [SCHAEFER & RENNEN, 2010; BEHERA & al. 2011]. The monoecious species *M. charantia* and *M. balsamina* produce edible fruits, and have been widely distributed as crops becoming naturalized throughout the tropics.

Cytotaxonomical, – genetic and – molecular analysis on *Momordica* spp. have been performed by: TRIVEDI & ROY (1973), BEEVY & KURICHAN (1996), LOMBELLO & FERREIRA PINTO-MAGLIO (2007), BHARATHI & al. (2011), KAUSAR & al. (2014) a.o. Karyo-taxonomical, – genetic and – geographical studies of Cucurbitaceae are mentioned since '70 years [AYYANGAR, 1976]. The results of these studies are presented in the table 1. Recently, a high-density, high-resolution genetic map was constructed for *M. charantia* by RAO & al. (2018), using Genotyping-by-Sequencing (GBS) Technology.

Table 1. Status of chromosome number and sequences in *Momordica* spp. [BHARATHI & al. 2011; RICE & al. 2015, The Chromosome Counts Database (CCDB)].

S.No.	**Species**	**Chromosome number & References**
1.	*Momordica balsamina* L.	*n* = 11 [JHA & TRIVEDI, 1989]; ***2n* = 22** [ROY & al. 1966]
2.	*M. charantia* L.	*n* = 11 [SHIBATA, 1962]; ***2n* = 22** [ROY & al. 1966]
3.	*M. cochinchinensis* (Lour.) Spreng.	*2n* = 28 [JHA & al. 1989]
4.	*M. cymbalaria* Fenzl ex Naudin	*n* = 8 [MEHETRE & THOMBRE, 1980]; *n* = 11 [BEEVY & KURIACHAN, 1996] ; ***2n* = 16, 22 [**BHARATHI & al. 2011]; ***2n* = 18** [CCDB]
5.	*M. denudata* (Thwaites) C. B. Clarke	*n* = 14 [BEEVY & KURIACHAN, 1996]
6.	*M. dioica* Roxb. ex Willd.	*n* = 14 [BEEVY & KURIACHAN, 1996] ***2n* = 28 [**ROY & al. 1966]; ***2n* = 42** [TRIVEDI, 1978]; ***2n* = 56** [ROY & al. 1966]
7.	*M. sahyadrica* Kattuk. & V. T. Antony	***2n* = 28**
8.	*M. subangulata* Blume	***2n* = 56**
9.	*M. tuberosa* Dennst. ex Miq. (syn. of *M. dioica* Roxb. ex Willd.)	*n* = 11 [AYYANGAR & SAMPATH-KUMAR, 1978]; ***2n* = 22** [AYYANGAR, 1976]

Referring to *Momordica* genus, RENNER and PANDEY (2013) presented also the status of sequences after SCHAEFER & RENNER, 2010, as follows (Table 2).

Table 2. Status of sequences in *Momordica* spp. [SCHAEFER & RENNER, 2010].

S.No.	Species	Sequences
1.	*Momordica balsamina* L.	HM367595, GQ163349
2.	*M. charantia* L.	DQ501269, HE585488 [SCHAEFER & RENNER, 2010; LIAO & al. 2012]
3.	*M. cochinchinensis* (Lour.) Spreng.	GQ163379, GQ163256
4.	*M. cymbalaria* Fenzl ex Naudin	An ITS sequence from an Indian specimen, Karuppusamy 28631 from Andhra PRADESH (ALI & al. 2009, GQ183046) is available and it is identical to sequences from Africa [SCHAEFER & RENNER, 2010]
5.	*M. denudata* (Thwaites) C.B. Clarke	SCHAEFER & RENNER (2010) generated sequences from THWAITES 28 (K), collected from Sri Lanka, GQ163385, GQ163262
6.	*M. dioica* Roxb. ex Willd.	GQ163389, GQ163387
7.	*M. sahyadrica* Kattuk. & V. T. Antony	No published sequences available [RENNEN & PANDEY, 2013]
8.	*M. subangulata* Blume	GQ163451, GQ163332

Origin and distribution of *Momordica charantia*

Origin. *M. charantia* is native to the Old World tropics. It is possibly domesticated in India and southern China. It is now found naturalized in almost all tropical and subtropical regions, being now pantropical. It is an important market vegetable in southern and eastern Asian and wild and cultivated populations can be found in India, Sri Lanka, Vietnam, Thailand and Malaysia, S. China and tropical Africa [PROTA, 2014]. It is belived that *M. charantia* was introduced into America from West Africa with the slave trade. In the West Indies, *M. charantia* was first recorded in Puerto Rico in 1885 (US National Herbarium). By the end of XIXth century, local cultivars originally from Asia were recorded on small scale cultivations in tropical America and the South United States (PROTA, 2014. PROTA4U web database. GRUBBEN G. J. H. & DENTON A. O. (Eds.), Wageningen, Netherlands: Plant Resources of Tropical Africa; http://www.prota4u.org/search.asp.).

Distribution. *M. charantia* is widely distributed throughout tropical and subtropical regions on all continents. It appears to be native to the African and Australian continents, but its actual origin has been obscured by its spread as a food crop. Currently it can be found cultivated and naturalized in North, Central and South America, the West Indies and on several islands in the Pacific Ocean (after ISC). The original place of domestification of *M. charantia* is unknown or unclear. The putative areas for domestification of *M. charantia* proposed by various workers include southern China, eastern India or eastern Asia [WALTERS & DECKER-WALTERS, 1988]. In the synthetic distribution table and map (https-www.cabi.org-isc-distribution-map) from *Invasive Species Compendium* (*ISC*), at 10. January 2020 (including the updated data presented in 2014 by J. ROJAS-SANDOVAL and P. ACEVEDO-RODRÍGUEZ, from Botany-Smithsonian NMNH Department, Washington DC, USA), there are presented the situation in Africa, Asia, North America, South America and Oceania.

Momordica charantia L. in Romania

M. charantia was known in 1990'years, when some seeds have been provided from Nepal by prof. dr. biologist Ovidiu BOJOR, mentioned by dr. STOIAN(OV) in *Sănătatea plantelor*, 2002, **48**(5): 9. Since 1990 up to the present, *M. charantia* plants was continually cultivated under opened-greenhouses at Romanian S. C. HOFIGAL EXPORT IMPORT S.A., which is manufacturing natural (bio)products from many pharma- and nutraceutical plants, among them *M. charantia*. In parallel with their specific cultivation of *M. charantia*, they are performed researchs in national projects like PN-II-PT-PCCA-2013-4-0995-160/2014 "Multifunctional and innovative products for safe and bioenhanced functional food from newly cultivated plants in Romania" developed at ICECHIM (INCDCP) Bucharest [ŞESAN, 2017; ŞESAN & al. 2018]. After the official published data from the Research-Development Station of Vegetables Buzău, under the egide of Academy of Agricultural Sciences and Forestry (AASF), the beginning of the bitter gourd crop started in the year 1992, when it have been brought some seeds from Tibet and Nepal, and has been started a crop which produced along the time 5 cultivars, among them Rodeo cv. (patent omologated by dr. engr. C. Vânătoru). Production was in greenhouse 60 t/ha and in the field 25 t/ha. At present *M. charantia* is evaluated as the most profitable crop in Romania. It was published a CD dedicated to the crop technology of bitter gourd under the condition of South Romania, at Buzău R-D Station for Vegetables (details in CĂPĂŢÎNĂ G. *Cultura castravetelui antidiabet*, CD, RENTROP & STRATON). Another project "High valorization of *Momordica charantia* L. (bitter gourd) for obtaining food functional ingredients with hypoglycemic effect" was developed by ONISEI (2016-2017) in the frame of Bioresources Research Institute of AASF. After 2000, it is important to mention some PhD thesis performed at the University of Agronomical Sciences and Veterinary Medicine Bucharest, having as research purpose the species *M. charantia* in order to establish its biomorphological features [STOIAN(OV), 2001], biotechnologies for obtaining some bioproducts based on medicinal plants (*M. charantia*) and fruits or approaches on leaf and stem anatomy of *M. charantia* [SĂVULESCU & HOZA, 2010]. After 2006, there were initiated approaches on *M. charantia* in the Western part of Romania, mainly at the University "Aurel Vlaicu" Arad [CRIŞAN & HĂLMĂJAN, 2007] and at University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca [KESERŰ & al. 2018].

Main biological activities of *Momordica charantia*

Momordica charantia L. has multiple benefic actions medicinal and nutraceutical, among them: (i) anti-inflammatory; (ii) anticancer/antimutagenic/antitumoral; (iii) anxiolytic, antistress, immuno-modulatory effects; (iv) antioxidant; (v) antimicrobial, antibacterial, antifungal, antiviral / anti-HIV; (vi) antiparasitic, antimalaria; (vii) beneficial for skin, antipsoriasis, anti-wound healing; (viii) anti-cardiovascular diseases, hypotensive, blood detoxifying; (ix) hepatoprotective; (x) protective for eyes diseases, cataracta a.o.; (xi) analgesic, antipyretric / febrifug; (xii) effects on reproductive activity, abortifacient and, especially, (xiii) antidiabetic, anti-obesity, hypoglycemic effects; (xiv) fungicide effect against phytopathogens; (xv) insecticide/ larvicidal/ pupaecidal effect against plant pests and others. A scheme of biological actions of *Momordica charantia* are presented in Figure 2.

Conclusions

M. charantia crop has started in Romania in years '90, when some seeds of the plant have brought from Nepal, Tibet, have been sown and aclimatized for their benefits, especially in horticulture, medicine, therapy health security a.o.

It has been accomplished a synthetic documentary study consisting in: botanical characteristics, plant description, taxonomy, nomenclature, plant origin and distribution, main benefic actions of *M. charantia* a.o.

Specific aspects of the *M. charantia* plant/crop (Plate I-II) are provided from opened-greenhouses of S.C. HOFIGAL EXPORT IMPORT S.A., which developed research activities in the national project PN-II-PT-PCCA-2013-4-0995-160/2014 "Multifunctional and innovative products for safe and bioenhanced functional food from newly cultivated plants in Romania" (2013-2017). *M. charantia* plants were deposited as Vauchers BUC Nr. 408946-408950 at the Botanical Garden of the Bucharest University-Romania.

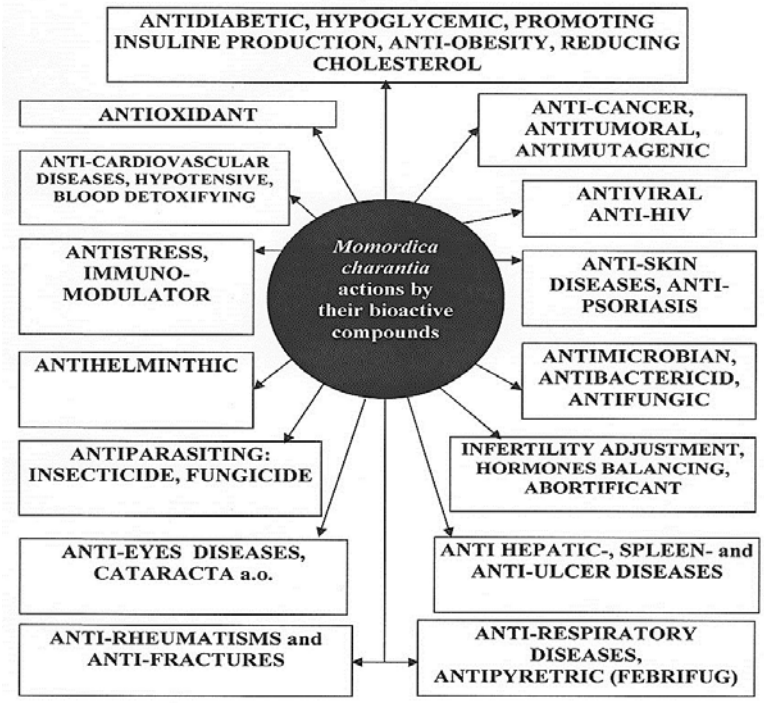

Figure 2. Main medicinal importance of *Momordica charantia*.

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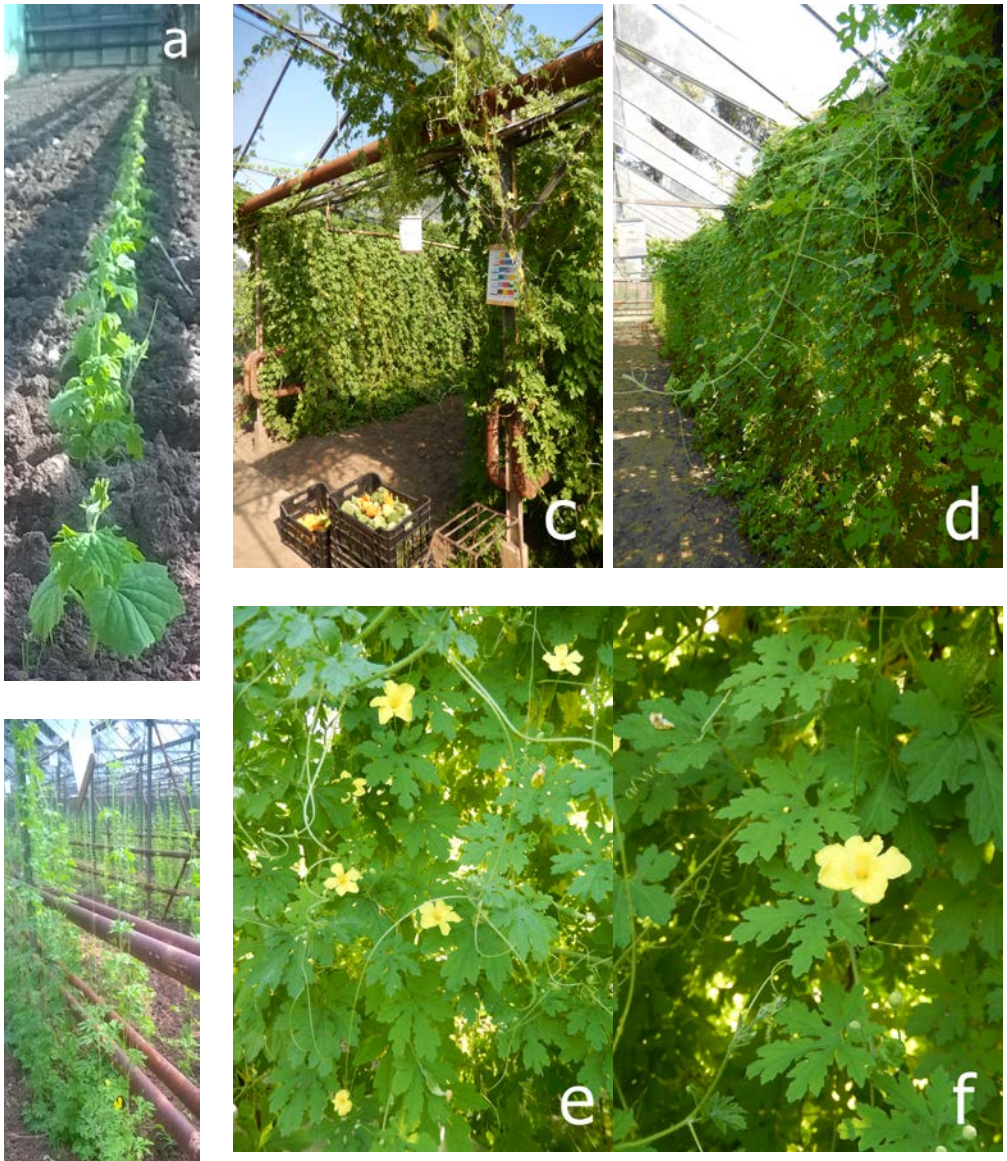
PLATE I



Momordica charantia characters in the crop at S.C. HOFIGAL EXPORT IMPORT S.A. (Vauchers BUC 408946-408950) under the opened greenhouse: **a**. crop after planting at 08.06.2016 and **b**. later at 05.07.2016; **c**. experimental plot at the finishing of experiment (06.09.2016); **d**. liane plants developed on the trellis; **e**, **f**. crop of bitter melon in flower with coiling stems, trendils, and palmate-divided leaves (photos: A. F. Popescu: **a**. 08.06.2016; **b**. 05.07.2016 and T. E. Şesan: **c**. 06.09.2016; **d**. 25.07.2016; **e**. and **f**. 06.09.2016).

PLATE II

♀ **flowers**

♂ **flowers**

Female flowers (up) and masculine flowers (down) in the first line of the plate; Fruits of *Momordica charantia* at different ages (**a-d**) and seeds (**e-f**) (photos: T. E. Şesan, 06.10.2016).

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THE FEATURES OF GROWTH, DEVELOPMENT AND CULTIVATION OF *NANDINA DOMESTICA* THUNB. UNDER THE CLIMATIC CONDITIONS OF THE REPUBLIC OF MOLDOVA

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Abstract: The article describes the bioecological features, the technology of propagation and cultivation of *Nandina domestica* Thunb. under the climatic conditions of the Republic of Moldova. The results of the research have shown that the optimal way of obtaining high-quality planting material is generative propagation, by sowing freshly cleaned seeds, in autumn, in a substrate consisting of forest humus and compost made from plants. The coefficient of uniformity and the germination capacity of seeds sown in autumn at a depth of 2-3 cm was 2-2.7 times higher than that obtained from sowing in spring. The planting material obtained after sowing in autumn is more vigorous than that obtained after sowing in spring, with a fasciculate and well-developed root system.

Keywords: Cultivation, development, *Nandina domestica* Thunb., propagation.

Introduction

The main objective of botanists has been the intensification of the activity of identification and mobilization of new plants from the spontaneous flora, as well as from the exotic one. The world flora provides us with a huge range of woody plants, which can be used to obtain benefits in various sectors of the national economy. In this context, we consider *Nandina domestica* Thunb., commonly known as nandina, heavenly bamboo or sacred bamboo, as a precious shrub with high potential for cultivation in the Republic of Moldova. Heavenly bamboo is a species in the family *Berberidaceae* Juss., which includes 14 genera and about 650 species, occurring in the temperate zone of North America and Eurasia, as well as in tropical and subtropical zones of Asia, it is successfully cultivated in the Crimea, the Caucasus and in the subtropical areas of the former Soviet Republics [DEREVIA I CUSTARNICHI SSSR, 1954]. In the Republic of Moldova, 37 species and cultivars of 3 genera of this family, namely *Berberis* L., *Mahonia* Nutt., *Nandina* Thunb., are cultivated currently [PALANCEAN, 2017, PALANCEAN & COMANICI, 2009]. In the "Alexandru Ciubotaru" National Botanical Garden (Institute) (NBGI), Chisinau, *Nandina domestica* Thunb. was introduced in 2011 from Nikitsky Botanical Garden, Yalta, Ukraine. The seeds were received by international seed exchange. In the Republic of Moldova, this species is very rarely found in green spaces, only in NBGI and maybe in the private gardens of some amateur horticulturists.

The purpose of our research has been to identify the bioecological features, the technology of propagation and cultivation of newly introduced plants, under the conditions of the Republic of Moldova.

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Materials and methods

The research was carried out in 2017-2020 in the plant nursery of the Dendrology Laboratory of the "Alexandru Ciubotaru" National Botanical Garden (Institute). The plants of *Nandina domestica* Thunb., from which the annual shoots were taken in 2 periods – March-April and June-July, and the fruits were harvested at the end of October, served as research subjects. The lignified cuttings were prepared in March and planted in cold frames in April. The semi-lignified cuttings were taken and prepared in June-July, when the intense growth of the shoots takes place, they are little lignified, but, when choosing the time to take cuttings, the climatic conditions under which they developed should be taken into account. The cuttings were cut and prepared with well-sharpened tools, in the morning, as fast as possible and at the optimal time for collecting them. The cuttings were treated with 0.01% solutions of $KMnO_4$ and IBA for 16 and 24 hours according to the methodology [HROMOVA, 1980]. The cuttings treated with distilled water served as control. The harvested fruits were soaked in water for 24 hours and the seeds were subsequently extracted. The freshly cleaned seeds were divided into 2 parts. Some of these seeds were sown in autumn in well-loosened soil at a depth of 2-3 cm. Another part of these seeds was stratified for 3-4 months. To determine the germination percentage, three repetitions (V1, V2, V3) of 100 seeds were performed in spring and autumn. Phenological observations on the growth of 6-7 year old mother plants and seedlings of nandina in the first growing seasons were made according to the method developed at the Moscow Botanical Garden [METODICA FENOLOGHICESCHIH NABLIODENII V BOTANICESCHIH SADAH SSSR, 1979] and perfected by Dr. hab. A. Palancean [PALANCEAN & COMANICI 2009].

Results and discussions

Nandina domestica Thunb. (nandina, heavenly bamboo or sacred bamboo) of the *Berberidaceae* family, is a species native to China and Japan. Nandina or heavenly bamboo, in its native country, is a small tree, 5-6 m tall, evergreen, with moderate growth rate. It also grows well on slopes. The heavenly bamboo plants introduced under the climactic and soil conditions of our country are evergreen shrubs with slow growth rate and reach up to 3 m in height. The root system is superficial. In the first 1-2 years, the annual shoots of seedlings obtained generatively were affected in winter, but in spring, the plants regenerated by growing new shoots from the root collar. The bark of young branches is initially light brown and then it becomes gray-brown and longitudinally ridged. The buds have an elongated shape, are pointed at the tip and are, on average, 1 cm long. *Nandina* is appreciated as an ornamental plant particularly due to the loose and airy crown, the colour of the foliage in early spring and late autumn, its aspect during flowering, fruit ripening and the bright colour of the fruits in late autumn. The leaves are imparipinnate compound. The petiole of the compound leaf was 10-15 cm long, and that of the leaflets – only 1-3 cm long. The flowers are white, 6 mm in diameter and are grouped in panicle inflorescences (Figure 1). The length of the inflorescences varied between 20 and 40 cm, the average being 28 cm. The flowers produce nectar, which attracts pollinating insects, especially bees. It bears flowers in June-July, but the fruits ripen in the September-October. As a result of the phenological research, it has been found that the number of fruits in an inflorescence varied from 91 to 171 units depending on the weather conditions during the flowering and fruiting stages. The average

weight of 1000 fresh fruits was 250 g and depended directly on the weather conditions during fruit onto-morphogenesis. The diameter of the seeds was 6 mm. The fruits were bright red, 8 mm in diameter and remained on the branches until the first frosts (Figure 2).

Figure 1. *Nandina domestica* in the flowering stage.

Figure 2. *Nandina domestica* in the fruit ripening stage.

In the first 1-2 growing seasons, it is characterized by slow growth of annual shoots, but produces a lot of shoots from the root collar. The colour of the wood is yellow-brown. It grows better in partial shade, but if it is cultivated in sunny areas, it needs artificial shading during the periods with hot weather.

Based on the analysis of the germination capacity of seeds and the percentage of successful rooting of cuttings, we have come to the conclusion that the optimal method of propagation is the generative one. Thus, some of the freshly cleaned seeds were sown in autumn in well-loosened soil, to a depth of 2-3 cm. Others were stratified for 90-120 days. The germination capacity of the seeds sown in autumn in trenches constituted 40-45%,

depending on the weather conditions during the flowering and fruit development and if the cultivation technology had been followed throughout the growing season (Table 1).

Table 1. The germination capacity of heavenly bamboo seeds in different periods of the year.

Year	Variant	Sowing date	Number		Germination percentage, %	
			seeds, units	obtained plants, units	per variant	average
2018	V1	20.IV	100	30	30	21.7
	V2	20.IV	100	20	20	
	V3	20.IV	100	15	15	
2018	V1	26.X	100	50	50	45
	V2	26.X	100	40	40	
	V3	26.X	100	45	45	
2019	V1	17.IV	100	10	10	15
	V2	17.IV	100	20	20	
	V3	17.IV	100	15	15	
2019	V1	24.X	100	40	40	40
	V2	24.X	100	45	45	
	V3	24.X	100	35	35	

The germination capacity of seeds sown in autumn was 2-2.7 times higher in comparison with those sown in spring. The seeds sown in autumn germinated uniformly at the beginning of May, but those sown in spring germinated unevenly in the first half of June and had low germination percentage (15-21.7%) (Figure 3).

The seedlings obtained from seeds sown in autumn were more vigorous and with a more developed root system as compared with those obtained from seeds sown in spring**.**

The length of the first-order roots of the seedlings obtained from seeds sown in autumn, in the second growing season, varied between 16 and 18 cm. The height of these plants in the second growing season was 28-35 cm. In the seedlings obtained from seeds sown in spring, the length of the roots varied between 10 and 14 cm, and the height of the plants was only 14-18 cm (Figure 4). Some researchers recommend the vegetative propagation of heavenly bamboo by cuttings, layering and grafting on mahonia. In the framework of our research, the results of the attempts to propagate nandina by lignified and semi-lignified cuttings, untreated and treated with root stimulators, were zero. The seedlings obtained generatively, after the first growing season, were transplanted into containers in a substrate slightly enriched with plant residues and complex mineral fertilizers, keeping its acidity within the limits of 4.5-5 for further growth, following the cultivation technology (Figure 5 a, b, c).

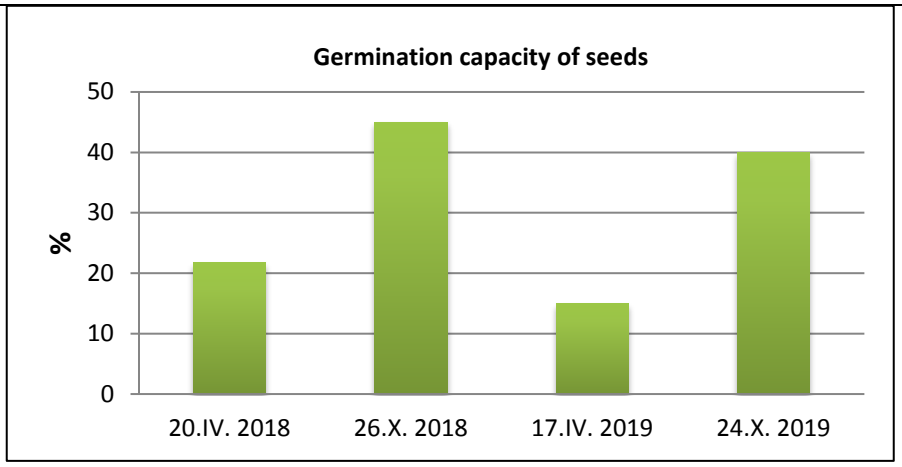

Figure 3. Germination capacity of seeds depending on the sowing period.

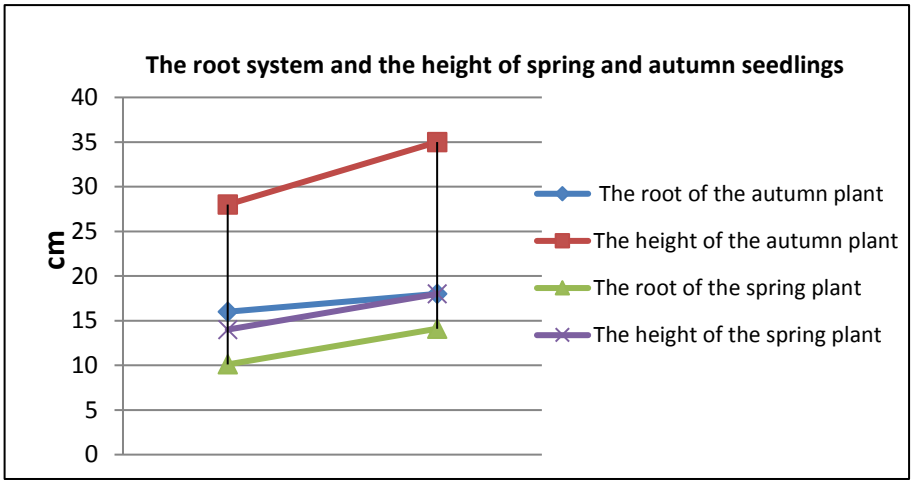

Figure 4. The growth rate of the root system and the height of *Nandina domestica* Thunb. plants in the second growing season.

Under the conditions of the Republic of Moldova, the only impediment to the cultivation of heavenly bamboo in the first 1-2 years was the gap between the lowest and the highest temperatures in winter and early spring. The cultivation of nandina in private gardens is profitable because it does not require special care. In early spring, it supports pruning the shoots by 1/3 of their length. The damaged branches are cut at the level of the root collar. During the whole growing season, additional complex mineral or fermented organic fertilizers are applied around the plant every month. If the plants are cultivated in containers, the substrate should be light, with pH=3.8-6.5, it should be changed every 2 years and fertilized with complex mineral substances (Figure 5). When transplanting plants into larger containers, the roots are slightly shortened. All parts of the plant are poisonous and it is

recommended to wash your hands after each contact with the plant. This shrub is decorative throughout the year and also has some medicinal uses, for example, it can be used as a raw material in antirheumatic, febrifugal and tonic remedies. Two varieties of high decorative value are known: *Nandina domestica* 'Alba' – a shrub with upright habit and white-cream fruits, *Nandina domestica* 'Royal Princess' – a 2-3 m tall shrub with more abundant flowering.

Figure 5. Seedlings obtained generatively in the second growing season (a – the root system of the seedlings obtained by sowing seeds in spring; b – the root system of the shoots obtained by sowing seeds in autumn; c – seedling grown in a container, evergreen).

It can be planted separately of in groups. Under the conditions of the Republic of Moldova, heavenly bamboo withstands frost, sometimes only annual vines partially freeze, it is undemanding in terms of soil fertility, withstands pruning and temporary lack of moisture.

Conclusions

As a result of bioecological and phenological research on *Nandina domestica* Thunb. plants (heavenly bamboo), it has been found that the growth and development rates match the annual cycle of our climate. Under the given conditions, it is an evergreen shrub with slow growth in the first growing seasons.

The optimal method of propagation of nandina (heavenly bamboo) was by sowing freshly cleaned seeds in autumn, in a light substrate enriched with plant residues and fermented organic fertilizers. The germination capacity of seeds sown in autumn at a depth of 2-3 cm was 2-2.7 times higher than that obtained from sowing in spring. The seeds sown in autumn germinate more evenly as compared with those sown into the soil in spring. The seedlings obtained from seeds sown in autumn are more vigorous and with a more developed root system as compared with those obtained from seeds sown in spring.

If heavenly bamboo is cultivated in containers, the substrate should be light, with pH varying between 3.8 and 6.5, and, every 2 years, it should be changed and fertilized with complex mineral substances.

Heavenly bamboo has been successfully studied can be recommended as an ornamental plant for landscaping in the central and southern districts of the Republic of Moldova, being decorative throughout the year, particularly due to the loose and airy crown, the colour of the foliage in early spring and late autumn, its aspect during flowering, fruit ripening, the bright colour of the fruits in late autumn.

It is cultivated in the foreground in small groups, along alleys, in all types of green spaces (except those near kindergartens, schools).

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VARIATION IN AGES OF TRANSPLANTS AFFECTS AGRO-MORPHOLOGICAL TRAITS OF SELECTED PEPPER LANDRACES FROM NIGER STATE, NIGERIA

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Abstract: Assessment of transplanting effects at different ages of seedling development on morphological and yield attributes in two landraces (SOMBO/MKW/2017 and NDAGBACHI/MKW/2017) of Nigerian pepper (*Capsicum* spp.) was studied during the planting season of 2017. The *Capsicum* landraces were obtained from local farmers in Mokwa, Niger State. The study was conducted at the experimental garden, Federal University of Technology, Minna, Niger State. Transplants were made at different ages (3, 4, 5 and 6 weeks after planting). The experimental design used was a Complete Randomized Design (CRD) with four replications of each treatment. Quantitative data obtained were pooled for analysis. Analysis of Variance (ANOVA) was used to compare the various mean values. Duncan Multiple Range Test (DMRT) post hoc test was used to separate the means. All values were considered significant at $p<0.05$. The result showed some interesting variations among the different ages of transplants; the results of the plant height at maturity and number of leaves for NDAGBACHI/MKW/2017 revealed that week 3 had the highest plant height (33.00 cm) at maturity and also the highest number (39.50) of leaves. However, these values were not significantly different ($p>0.05$) from all other weeks. The results for plant height at harvest and number of branches revealed that week 4 recorded the highest height (37.75 cm) at harvest and highest number (8.00) of branches, but is not statistically different ($p>0.05$) from other weeks. For SOMBO/MKW/2017, Week 4 recorded highest in terms of plant height (30.08 cm) at maturity, plant height (32.50 cm) at harvest and number of branches (9.25). These values were significantly different ($p<0.05$) from other weeks. For NDAGBACHI/MKW/2017, Week 3 produced the highest number of fruits/plant (28.00) but bears no significant difference ($p>0.05$) to all other weeks. However, for SOMBO/MKW/2017, Week 4 recorded the highest number of fruits/plant (35.00) and is significantly different ($p<0.05$) from week 3 and 6 but statistically the same with week 5. In both landraces, Week 3 produced the highest number of seeds/fruit, but is statistically the same ($p>0.05$) with other weeks. It is therefore concluded that variation in transplanting age tend to influence certain morphological and yield attributes in Nigerian pepper landraces. Also, 3 and 4 week old transplants seem to be the best age to produce more yields for NDAGBACHI/MKW/2017 and SOMBO/MKW/2017 respectively. Further research works should be done to test for other higher ages at transplanting and also for different landraces in order to arrive at a sound conclusion.

Keywords: *Capsicum*, landraces, NDAGBACHI/MKW/2017, SOMBO/MKW/2017, transplanting, yield attributes.

Introduction

Pepper is an economically important crop belonging to the night shade family Solanaceae and genus *Capsicum* often divided into pungent and non-pungent, [PICKERSGILL, 1997]. Pepper originated from Central and South America where it is still being cultivated [GRUBBEN & EL-TAHIR, 2004]. The genus consists of over 100 species and even more botanical varieties [FALUSI, 2007]. These include five domesticated

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species (*Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. pubescens* and *C. baccatum*). *C. annuum* and *C. frutescens* are the most recognized species grown in commercial quantities all over Nigeria [FALUSI & MORAKINYO, 2001; MADY & al. 2005]. These two species form an important ingredient in people's diet around the world. The major center of diversity is Brazil, where representatives of all the species are found [COSTA & al. 2009]. Despite being the center of origin and diversity of *Capsicum* species, little is known about the native species, particularly *C. chinense* Jacq., with great variability in the Amazon. They are considered the first spice used by humans and there is archaeological evidence of pepper and other fossil foods from as early as 6,000 years ago [HILL & al. 2013]. Pepper was introduced to Europe by Columbus and other early new explorers in the sixteenth century and cultivation spreads throughout the world [GREENLEAF, 1986].

Pepper is an important commercial crop grown for vegetable spice and value-added processed products [KUMAR & RAI, 2005]. It is an essential component of many foods adding flavour, colour, vitamins A and C and pungency and it is therefore, indispensable to world food industries. It can be used medically for the treatment of fevers, colds, indigestion, constipation and also as painkiller [DAGNOKO & al. 2013]. It is also used by security agencies to prepare tear gas. Peppers are an important source of nutrients in the human diet [SHETTY & al. 2013], and it can be consumed fresh or dried. They promote health benefits such as reducing obesity and diabetes [VASCONCELOS, 2016]. Pepper extracts are used in cosmetics and pharmaceuticals. Besides the use in feed as spice, pepper has also ornamental potential when grown in pots or gardens [BOSLAND & VOTAVA, 2012]. Proper age of transplanting, as an important cultural practice in pepper growing, is very important for maximum production of the crop.

Transplanting or indirect seedling is the process of growing seedlings in a greenhouse or other controlled environment before placing plants outdoors [AKINROTIMI & ANIEKWE, 2018]. The effect of transplant age on yield is an issue often investigated by growers to maximize production potential [VAVRINA, 1998]. LIPTAY (1987) reported that moisture, which is essential for top growth of plants, is absorbed by root hairs. Such important roots hairs are destroyed during transplanting. The older the plant the more extensive its root system and the greater the root hairs lost in transplanting. NORMAN (1977) in his study on the effect of age of transplants on hot pepper remarked that flowering, fruiting and harvesting were delayed by transplanting and therefore recommended 5-6 weeks old transplants for hot pepper production in Ghana. When transplants are thought to be too old, concerns are raised about their subsequent growth and yield potential. Young transplants (3 to 4 weeks) reduce production costs, but will need to be grown longer in the field to reach optimum yields [LESKOVAR & al. 1991; ORZOLEK & al. 1991].

Research works done in other countries show that both growth and yield in pepper are markedly influenced by differentially aged seedlings and transplanting date. Cultural factors, such as transplant age [MCCRAW & GREIG, 1986; WESTON, 1988], pruning [MCCRAW & GREIG, 1986], fertility [SUNDSTROM & al. 1994], influenced pepper yield. LEE & al. (2001) reported that seedling growth of chilies was greater with increasing age of transplants. They concluded that plant height, dry weight and the number of branches increased with seedling age. It was based on this premise that this research was set up to evaluate the effect of various transplanting ages on the growth and yield of two landraces of pepper in Niger State, Nigeria and to determine the best transplanting age for this crop.

Materials and methods

The Landraces of *Capsicum annuum* and *C. frutescens* were obtained from a local farmer in Kpaki, Mokwa Local Government area of Niger State. They were raised in planting trays before transplanting into planting bags at various weeks. Sixteen planting bags were obtained from Bida and labeled with the code of each landrace into which the corresponding landraces from the planting trays were transplanted. The experimental design used was a Complete Randomized Design (CRD) with four replications of each treatment. The ages of transplants were 3 weeks, 4 weeks, 5 weeks, and 6 weeks old after planting respectively. Seedlings were raised on well prepared nursery beds during the raining season of 2017. After sowing, the beds were watered. Transplanting was done early in the morning with four replicates of each landrace.

All the parameters investigated were assessed using the standard procedures of AKINYELE & OSEKITA (2006) as well as DAUDU & al. (2015) and AKINROTIMI & ANIEKWE (2018). The following parameters were studied.Plant height were taken at transplanting, maturity (100% flowering) and harvest; number of leaves and branches, number of leaves and branches of the two landraces was counted and the mean was recorded. Number of fruits/plant was obtained after the fruits harvested from the two landraces were counted from each treatment and the mean values recorded.Weight of fruits was done by random selection often fruits and each of them was weighed using an electronic weighing balance. Others include: number of seeds and Weight of 100 Seeds. Quantitative data obtained were pooled for analyses. Analysis of variance (ANOVA) was used to compare the various mean values. Duncan Multiple Range Post Hoc Test (DMRT) was used to separate the means. All values were considered significant at 95% confident level.

Results and discussion

The descriptive characteristics of the pepper landraces used in this study are presented in table 1; these include a hot chili (NDAGBACHI/MKW/2017) and long pepper with mild hotness (SOMBO/MKW/2017). The result obtained for the morphological parameters of NDAGBACHI/MKW/2017 is presented in table 2. The Analysis of Variance (ANOVA) showed that there is significant difference ($p<0.05$) for plant height at transplanting. The highest plant height (6.50 cm) was produced by Week 6; this value was significantly different ($p<0.05$) from all the other values. The lowest value (4.05 cm) was produced by Week 3. However, the highest plant height at maturity (33.00 cm) was found in Week 3 while the lowest value (23.13 cm) was found in Week 4; these values were significantly different from each other. In terms of plant height at harvest, the results showed that there was no significant difference ($p>0.05$) among the Weeks. The highest plant height (37.75 cm) was produced by Week 4 while the lowest plant height (27.13 cm) was produced by Week 6. Similarly, this highest value was significantly different from each all the other weeks. For number of branches, the results showed that no significant difference existed ($p>0.05$) between transplants at week 3, 4 and 5; but the values were significantly different from that of week 6 after transplant (2.75). Week 4 recorded the highest number of branches (8.00) while which was followed by week 3 and 5 (5.25 each). There is no significant difference ($p>0.05$) among week 3, 4 and 5 after transplanting in terms of number of leaves. However, transplant at 3 week old had the highest number of

leaves (39.50) while the lowest number of leaves (19.25) was produced by Week 6; these values were significantly different from each other.

The result obtained for the morphological parameters of SOMBO/MKW/2017 is also presented in table 2. There were significant differences ($p<0.05$) among the weeks in terms of plant height at transplanting. The highest plant height (4.53 cm) was found in Week 5; this value was statistically different ($p<0.05$) from all the other values. The lowest plant height (3.80 cm) was found in Week 3.

The highest plant height at maturity (30.08 cm) was recorded for Week 4 and lowest plant height (8.38 cm) was recorded from Week 6. For plant height at harvest, the results showed that there is no significant difference ($p>0.05$) in plants transplanted at week 3, 4 and 5. However, the highest plant height (32.50 cm) was found in Week 4 which was significantly different from the lowest (21.00 cm) obtained in Week 6. In terms of number of branches per plant, the highest number of branches (9.25) was produced by Week 4 while the lowest number of branches (4.75) was produced by Week 6; these values were significantly different from each other. The results for number of leaves per plant showed that there is significant difference ($p<0.05$) among the Weeks. The highest number of leaves (83.25) was recorded in Week 3; this value was statistically different from other Weeks. The lowest number of leaves (19.00) was recorded in Week 6.

The result obtained for the yield parameters of NDAGBACHI/MKW/2017 is presented in table 3. The highest weight of fruit (0.80) was recorded in the landrace transplanted at Week 5 after planting while the lowest (0.38) was recorded in the landrace transplanted at Week 6 after planting. These values are significantly different from each other. In terms of number of fruits/plant, Week 3 had the highest number of fruits (27.50) per plant but was not significantly different ($p>0.05$) from those obtained in week 4 transplants. However, Week 6 produced the lowest number of fruits/plant (2.75). These values were statistically the same with all other Weeks. For number of seeds/fruit, the result showed that week 3 transplants produced significantly highest number of seeds per fruit among all the other weeks. The highest number of seeds (41.20) was produced by Week 3 while the lowest number of seeds (30.20) was produced by Week 6. The weight of 100 seeds recorded highest in Week 5 (0.41 g) and lowest in Week 6 (0.20 g); these values were significantly different ($p<0.05$) from each other and those of other weeks.

The result obtained for the yield parameters of NDAGBACHI/MKW/2017 is also presented in table 3. The Analysis of Variance for the weight of fruits showed that there is no significant difference ($p>0.05$) among the Weeks. However, the highest weight of fruit (12.09 g) was produced by Week 3 and the lowest weight of fruit (7.97 g) was recorded in Week 6. These values were not significantly different ($p>0.05$) from all the other Weeks. In terms of number of fruits/plant, Week 4 had the highest number of fruits (35.00) per plant and was significantly different ($p<0.05$) from other Weeks. However, Week 6 produced the lowest number of fruits/plant (7.50). These values were statistically different ($p<0.05$) from other Weeks.

For number of seeds/fruit, the result showed that there is no significant difference ($p>0.05$) among the Weeks. The highest number of seeds (143.00) was produced by Week 3 while the lowest number of seeds (108.80) was produced by Week 5. These values were significantly different from each other. For weight of 100 seeds, the ANOVA showed that there is highly significant difference ($p<0.05$) between the Weeks. The highest weight (0.52 g) was produced by Week 3 while the lowest weight (0.40 g) was produced by Week 4. The values were significantly different from one another (Table 3).

The results obtained in terms of morphological and yield parameters in both pepper landraces (SOMBO/MKW/2017 and NDAGBACHI/MKW/2017) indicate that they were affected by various ages of transplants, this assertion is in line with that of AKINROTIMI & ANIEKWE (2018). However, the result is in contrary from that of VAVRINA & ARMBRESTER (2004) who found no effect of transplant age on yield (number and weight) in three of four harvests, but a significant yield increases at fourth harvest. The observed results in term of certain agro-morphological traits are in accordance with the report of SAFINA & al. (2006) who reported that 40 days (5 weeks) old transplants recorded the highest plant height, 50 days (6 weeks) old transplants exhibited best growth in terms of number of branches and number of leaves per plant. The results obtained in terms of plant height at transplanting indicate that plant height varies with age. The results obtained for plant height at maturity show that 3 week old transplants produced the highest plant height for NDAGBACHI/MKW/2017 and the 4 week old transplants produced the highest plant height for SOMBO/MKW/2017. For plant height at harvest, 4 week old transplants recorded the highest plant heights in both landraces. This result is contrary to that of SAFINA & al. (2006) who reported that 5 week old transplants recorded the highest plant height. This variation might be due to the differences in environmental factors and/or differences in growing seasons and even genetic factors. For number of branches per plant, 4 week old transplants produced the highest number of branches in both landraces. NORMAN (1977) who recommended 5-6 weeks old transplants for hot pepper production is in contrast to this finding, because 3 weeks old (for NDAGBACHI/MKW/2017) and 4 weeks old (for SOMBO/MKW/2017) transplants tend to perform better. These differences could be due to differences in environmental factors. For number of leaves, 3 weeks old transplants recorded the highest number in both landraces; this is in conformity with that reported by AKINROTIMI & ANIEKWE (2018).

The results obtained for weight of fruits and weight of seeds indicate that landraces transplanted at 3 weeks after planting (for SOMBO/MKW/2017) and 5 week after planting (for NDAGBACHI/MKW/2017) produced the highest weight of fruits and seeds. The younger transplants (week 3 transplants) in this study produced the highest number of seeds per fruit. The results obtained for number of fruits per plant is an indication that 3 week old (for NDAGBACHI/MKW/2017) and 4 week old (for SOMBO/MKW/2017) transplants produced the highest number of fruits. This observation is in line with the work of SHUKLA & al. (2011) who reported that middle aged transplants produced more number of fruits than the younger and older transplants. ADELANA (1983) also recommended that 3 and 4 weeks old transplants are the best for high yield of fruit of pepper (a Solanaceous plant). AKINROTIMI & ANIEKWE (2018) opined that transplanting green pepper from 1 to 3 weeks was suitable transplanting time, because during this stage, the plant can easily withstand shock due to transplanting, without shedding its leaf. The possible reason seems to be that in case of younger seedlings there was less storage of food needed for vegetative extension, whereas, older transplants were mature enough and restrained vegetative extensions, thus producing lower yields.

Table 1. Description of the Pepper (*Capsicum* spp.) genotypes used in this study.

Landrace number	Source	Local name	Botanical name	Botanical description
SOMBO/MKW/2017	Mokwa	Sombo	*Capsicum annuum*	Medium-sized annual plant, long pointed and pendant fruits with hot taste, one pedicel per node.
NDAGBACHI/MKW/2017	Mokwa	Ndagbachi	*Capsicum frutescens*	A small-sized perennial plant, with very hot taste, three to five pedicels per node.

Table 2. Morphological parameters of the selected landraces of *Capsicum* spp.

Age at transplant	Height/cm			Morphology at maturity	
	Transplanting	Maturity	Harvest	Number of branches	Number of leaves
NDAGBACHI/MKW/2017					
Week 3	4.05±0.05^{a}	33.00±4.00^{b}	33.75±1.25^{a}	5.25±2.25^{b}	39.50±4.50^{b}
Week 4	4.18±0.33^{b}	23.00±2.00^{a}	37.75±0.75^{b}	8.00±1.00^{b}	33.00±1.50^{b}
Week 5	6.00±0.50^{b}	29.75±6.25^{ab}	32.05±8.45^{a}	5.25±1.75^{b}	38.25±6.75^{b}
Week 6	6.50±0.75^{c}	23.13±3.88^{a}	27.13±3.03^{a}	2.75±0.25^{a}	19.25±6.25^{a}
Total	5.18±0.45	27.22±2.30	32.67±2.24	5.31±0.91	32.50±3.61
SOMBO/MKW/2017					
Week 3	3.80±0.50^{a}	21.38±0.13^{c}	28.25±4.75^{b}	6.50±0.50^{ab}	83.25±11.75^{c}
Week 4	4.35±0.00^{b}	30.08±7.93^{d}	32.50±7.50^{b}	9.25±0.25^{c}	60.50±3.00^{b}
Week 5	4.53±0.73^{c}	17.13±0.88^{b}	24.25±0.75^{b}	7.25±0.75^{b}	64.00±4.00^{b}
Week 6	3.95±0.5^{d}	8.38±0.13^{a}	21.00±1.00^{a}	4.75±0.25^{a}	19.00±2.00^{a}
Total	4.16±0.20	19.24±3.22	26.50±2.35	6.94±0.64	56.69±5.19

Values are means ± standard error, values followed by the same superscript on the same column is not significantly different at p>0.05 tested by DMRT.

Table 3. Yield parameters of the selected landraces of *Capsicum* sp.

Age at transplant	Number of fruits/plant	Weight of fruits (g)	Number of seeds/fruit	Weight of 100 seeds (g)
NDAGBACHI/MKW/2017				
Week 3	27.50±2.00^{c}	0.56±0.07^{b}	41.20±1.85^{b}	0.25±0.00^{b}
Week 4	23.75±2.25^{c}	0.77±0.04^{c}	37.40±2.68^{ab}	0.38±0.00^{c}
Week 5	17.50±3.00^{b}	0.80±0.06^{c}	37.80±5.18^{ab}	0.41±0.00^{d}
Week 6	13.75±0.75^{a}	0.38±0.06^{a}	30.20±4.50^{a}	0.20±0.00^{a}
Total	20.63±0.91	0.63±0.05	36.65±1.97	0.31±0.20
SOMBO/MKW/2017				
Week 3	11.25±0.75^{b}	12.09±2.06^{a}	143.00±11.47^{b}	0.52±0.00^{d}
Week 4	35.00±2.50^{d}	8.91±1.65^{a}	127.40±18.76^{b}	0.40±0.00^{a}
Week 5	25.00±1.50^{c}	8.51±1.26^{a}	108.80±21.40^{a}	0.42±0.00^{c}
Week 6	7.50±0.50^{a}	7.97±1.75^{a}	115.40±14.55^{a}	0.41±0.00^{b}
Total	19.69±0.01	9.37±0.86	123.65±8.34	0.44±0.00

Values are means ± standard error, values followed by the same superscript on the same column is not significantly different at p>0.05 tested by DMRT.

Conclusion and recommendation

It can be concluded that Although, different transplanting ages tend to favour different agro-morphological traits, the best ages for transplanting of both the pepper landraces SOMBO/MKW/2017 and NDAGBACHI/MKW/2017) for optimum yield attribute preferred by growers (number of fruits per plant) are the 3-4 week after planting. Also, more specifically, 3 and 4 week old transplants seem to be the best age to produce more yields for NDAGBACHI/MKW/2017 and SOMBO/MKW/2017 respectively. It is therefore recommended that appropriate timing for transplanting, as an important cultural practice, should be considered for optimum production of the selected pepper landraces in Niger State, Nigeria.

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EFFECT OF WATER STRESS ON PHYSIOLOGICAL PARAMETERS OF BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA* (L.) VERDC.) ACCESSIONS

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Abstract: In order to evaluate the effect of water stress on physiological parameters of bambara groundnut, twenty-eight accessions were evaluated at the Experimental garden of the Department of Biological Sciences, Federal University of Technology, Minna, Niger State Nigeria. They were subjected to two treatments, water stressed and non-water stressed. The accessions were sown in planting bags and arranged in a randomized complete block design with three replicates. Significant differences in all physiological parameters were observed between the bambara groundnut accessions in both the water stressed and the non-water stressed treatment. Relative water content, chlorophyll a, chlorophyll b and total chlorophyll content was higher in non-water stressed plants than water stressed plants, although relatively high water content, chlorophyll a, b and total chlorophyll contents were still observed in stressed plants. It was also observed that bambara groundnut accessions explored drought tolerant mechanisms by closing their stomata to reduce water loss.

Keywords: accessions, bambara groundnut, chlorophyll, drought, relative water content, water stress.

Introduction

Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) – Family *Fabaceae*, is a legume plant native to Africa [ADU-DAPAAH & SANGWAN, 2004]. It is the third most commonly eaten legume after Cowpea (*Vigna unguiculata*) and Peanuts (*Arachis hypogea*) and a major source of protein for resourced poor farmers in Nigeria [ABEJIDE & al. 2017]. It is an annual crop grown for the purpose of its seeds which are produced in pods under the ground. Bambara groundnut exists as landraces, rather than cultivars, and consists of several genotypes resulting in their ability to tolerate different types of stress (both biotic and abiotic) in low input Agricultural Systems [LINNEMANN & CRAUFURD, 1994]. Landraces have recognizable morphological features such as testa colour that can be used to identify them. The Landraces are named mostly based on the testa colour or the place where they are grown or collected [LACROIX & al. 2003].

Rainfall is low and erratic in the semi-arid parts of Nigeria, where the production and consumption of bambara groundnut are well established [ABEJIDE & al. 2018]. Consequently drought can be a limiting factor in the production of rainfed crops in these regions [AZAM-ALI & al. 2001]. Limited evidence exists on the degree and mechanism of resistance of bambara groundnut to drought. MALHOTRA & SAXENA (2002) observed differences in the response of two bambara groundnut landraces and attributed that to climatic conditions in the area of collection. In order to improve the yield and to fully

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exploit the potential of bambara groundnut, a more thorough understanding of the mechanisms of its response to water stress is needed.

Materials and methods

Source of seeds

The seeds used for the study were collected from farmers in seven States in Northern Nigeria such as Niger, Kogi, Plateau, Kaduna, Nassarawa, Adamawa and Jigawa States. Some seeds were also collected from National Centre for Genetic Resources and Biotechnology (NACGRAB) Ibadan, Nigeria. The seeds were sown at the Department of Biological Sciences Experimental garden, Federal University of Technology Minna, Niger State. Minna is located between latitude 90˚31′ and 90˚45′ North and longitude 60˚31′ and 60˚45′ East of the equator. The area falls within the Southern Guinea savannah vegetation zone of Nigeria with an annual precipitation varying from 1,100-1,600 mm, mean temperature between 21 °C and 36.5 °C and relative humidity between 50 to 61% (The Nigerian Congress, 2007).

Experimental design

Bambara groundnut accessions were grown in two different treatments Non Water stressed (NST) and water stressed (ST) condition. Plants were rain fed and received water at least twice in a week throughout the experiment except for the stressed plants which were deprived of water for 14 days during the flowering stage by transferring plants to a shade house made of transparent polyvinyl ceiling. The accessions were grown in planting bags in a randomized complete block design with five replicates. Two seeds were sown per bag and the bags were given a spacing of 30 x 30 cm (inter and intra row spacing) and later thinned to one at 2 weeks after planting. The following physiological parameters were determined after stress period both in the stressed plants and non-water stressed plants, relative water content, chlorophylls a, b, total chlorophyll content and stomata opening.

Relative water content (RWC)

RWC was determined following the method of VURAYAI & al. (2011). Five leaf discs from leaves of three tagged plants per replication were cut using a cork borer (about 11 mm diameter). The leaf discs were placed in pre-weighed vials, sealed and reweighed to derive their fresh weight (FW) before being placed in petri dishes lined with two layers of germinating paper saturated with deionized water. This was sealed with tape to prevent evaporation and left overnight under a light source to allow discs to re-hydrate to their turgid weight (TW). Their dry weight (DW) was obtained after overnight drying at 80 ºC for 48 h. The leaf RWC was then measured and calculated according to TURNER & BEGG (1981) as:

$$RWC\ (\%) = [(FW-DW) / (TW-DW)] \times 100\%$$

Stomata opening

Three leaves of bambara groundnut from each treatment were harvested at about 9 a.m. in the morning. Nail polish was applied on the clean dry leaf and then celotape was placed on it. It was then allowed to dry and peeled off. Afterwards, the celotape with the peeled polish was mounted on a slide and then viewed with a microscope (x40-x100).

Leaf chlorophyll content

Leaf disks of about 0.25 g were used for determination of actual leaf chlorophyll content by photometric methods as described by PORRA & al. (1989). Chlorophyll was extracted from the leaf tissue using a buffered 80% aqueous acetone solution and absorbance was measured on the supernatant by a UV 160 IPC spectrophotometer. Chlorophyll content was expressed in Xg chl.g/Fwt, where Fwt denotes fresh weight. Chlorophyll a, b and total chlorophyll was determined using the below formulas:

Chlorophyll a = $10.3D_{663} - 0.918D_{644}$

Chlorophyll b = $19.7D_{644} - 3.8D_{633}$

Total Chlorophyll = $6.4Q_{633} + 18.8D_{644}$

Where D_{663} = Value of absorbance at wavelength 663

D_{644} = Value of absorbance at wavelength 644

D_{633} = Value of absorbance at wavelength 633

Results

Relative water content

There were significant differences observed in the relative water content of the bambara groundnut accessions in both stressed and non-stressed plants. The highest relative water content was observed in accession (NGB-01491) in both stressed and non-stressed plants (72.73, 75) and the least relative water content was observed in accession (NGR-KG-02-C) in both water stressed and non-stressed plants (42.36, 48). It was observed that the bambara groundnut accessions were still able to maintain high relative water contents despite the water stress (Figure 1).

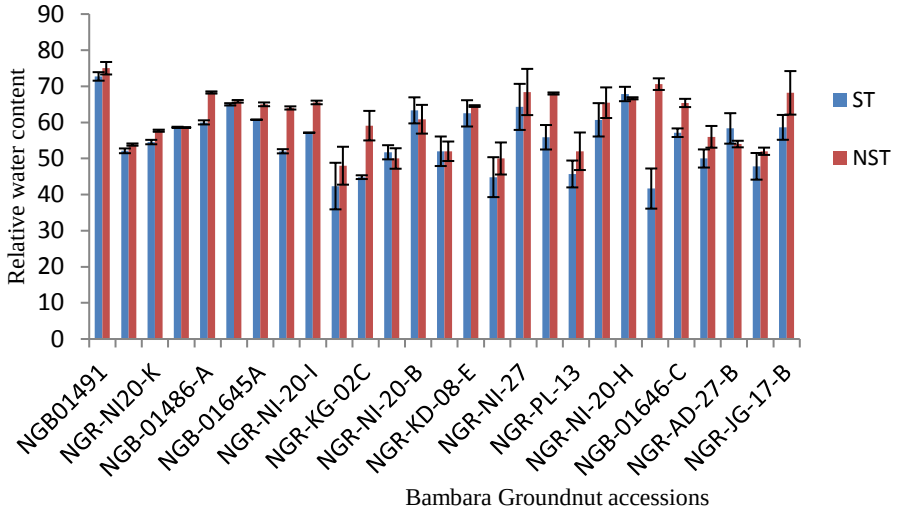


Figure 1. Relative water content of Water stressed and Non-water stressed Bambara Groundnut accessions.

Chlorophyll content

There were also significant differences observed in chlorophyll a, chlorophyll b and total chlorophyll content amongst the bambara groundnut accessions in both stressed (ST) and non-water stressed (NST) plants (Table 1). The highest chlorophyll a content in the stressed plants was observed in accession NGR-NI-25-A (10.90 g). It was not significantly different from accessions NGB-01645-A, NGR-NI-20-J, NGR-AD-27-B and NGR-JG-17-A but significantly different from all other accessions. The lowest was observed in accession NGR-NS-15 (4.23 g) and it was significantly different from all other accessions except NGR-NI-27, NGB-01493, NGR-NI-20B, NGR-PL-12, NGB-01496, NGR-NI-20H and NGB-01646-B. In the non-water stressed plants, the highest chlorophyll a content was observed in accession NGR-PL-12 (12.50 g) and it was not significantly different from accessions NGR-NI-27 and NGR-NI-20-J but significantly different from all other accessions, while the lowest was observed in accession NGR-NI-20-I (7.15 g) and it was not significantly different from accession NGR-KG-01 but significantly different from all other accessions.

The highest chlorophyll b content in the stressed plants was observed in accession NGB-01646-C (23.94 g) and the lowest in accession NGB-01493 (10.37 g) which was significantly different from all other accessions. In the non-stressed plants the highest chlorophyll b content was observed in accession NGR-AD-27-B (23.57 g). It was not significantly different from accessions NGR-JG-17-A, NGB-01486-A, NGR-KG-02-C but significantly different from all other accessions while the least chlorophyll b content was observed in accession NGR-NI-20-I (19.78 g) and it was significantly different from all other accessions.

The highest total chlorophyll content in the stressed plants was observed in accession NGB-01645-A (34.02 g) and the least in accession NGB-01493 (15.36 g) and it was significantly different from all other accessions except accession NGR-NS-15. In the non-stressed plants the highest total chlorophyll content was observed in accession NGR-NI-20-H (37.41 g) and it was significantly different from all other accessions. The least total chlorophyll content was observed in accession NGR-NI-20-I (27.19 g) and it was significantly different from all other accessions. Chlorophyll a, b and total chlorophyll contents had higher values in the non-water stressed plants compared to the stressed plants for most of the accessions.

Stomata opening

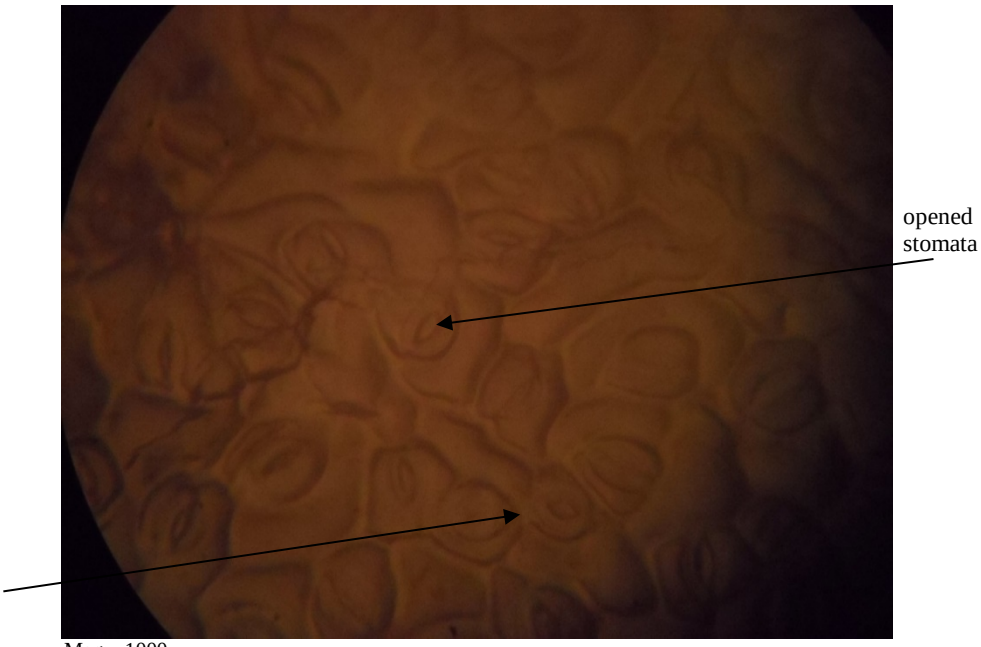
The stomata opening of the bambara groundnut accessions in the non-water stressed plants were observed to be opened but in the stressed plants, the stomata opening were observed to be closed. Figure 2 is the stomata opening of bambara groundnut accession in the non-water stressed plants showing opened stomata, while figure 3 is the stomata opening of the bambara groundnut accession in the stressed plants showing closed stomata.

Table 1. Mean Values of Chlorophyll content measured in water stressed (ST) and non-water stressed (NST) conditions.

S/No	Accessions	Chlorophyll a		Chlorophyll b		Total Chlorophyll	
		ST	NST	ST	NST	ST	NST
1	NGB01491	8.35±0.00^{b}	11.98±0.01^{b}	21.31±0.04^{b}	22.64±0.01^{b}	29.38±0.04^{b}	35.58±0.01^{bc}
2	NGB-01493	6.58±0.02^{ab}	9.53±0.00^{b}	10.37±0.03^{a}	22.61±0.00^{b}	15.36±0.00^{a}	33.14±0.00^{b}
3	NGR-NI20-K	7.44±0.00^{b}	11.45±0.01^{b}	20.37±0.03^{b}	22.84±0.02^{b}	28.14±0.03^{b}	35.03±0.03^{bc}
4	NGB-01311	7.96±0.01^{b}	11.49±0.00^{b}	20.57±0.03^{b}	22.99±0.13^{b}	28.54±0.01^{b}	34.88±0.01^{bc}
5	NGB-01486-A	9.75±0.01^{b}	11.63±0.17^{b}	22.29±0.04^{c}	23.30±0.01^{c}	32.22±0.03^{bc}	35.17±0.02^{bc}
6	NGR-NI-23-C	9.45±0.02^{b}	11.93±0.01^{b}	22.43±0.04^{c}	22.90±0.01^{b}	31.27±0.03^{b}	35.08±0.02^{bc}
7	NGB-01645A	10.19±1.09^{c}	10.98±0.02^{b}	22.92±0.02^{c}	22.98±0.01^{b}	34.02±0.00^{c}	34.49±0.02^{bc}
8	NGR-NI-18	7.65±0.00^{b}	9.76±0.00^{b}	21.39±0.03^{bc}	21.89±0.01^{b}	29.60±0.02^{b}	33.12±0.02^{b}
9	NGR-NI-20-I	7.33±0.00^{b}	7.15±0.00^{a}	20.77±0.01^{b}	19.78±0.00^{a}	28.22±0.02^{b}	27.19±0.00^{a}
10	NGR-NI-22	7.29±0.01^{b}	10.81±0.00^{b}	20.54±0.03^{b}	22.62±0.01^{b}	27.43±0.02^{b}	34.00±0.01^{bc}
11	NGR-KG-02C	7.75±0.01^{b}	10.65±0.^{b}	21.62±0.02^{b}	23.50±0.04^{c}	28.75±0.02^{b}	34.50±0.05^{bc}
12	NGR-KG-01	9.45±0.03^{b}	8.97±0.02^{a}	22.90±0.05^{bc}	21.97±0.15^{b}	32.13±0.06^{bc}	32.16±0.03^{b}
13	NGR-NI-20-B	6.41±0.01^{ab}	9.96±0.01^{ab}	18.67±0.02^{b}	22.43±0.02^{b}	25.03±0.02^{b}	33.31±0.04^{b}
14	NGR-PL-12	6.98±0.01^{ab}	12.50±0.00^{c}	20.19±0.02^{b}	22.65±0.07^{b}	27.54±0.03^{b}	35.64±0.04^{bc}
15	NGR-KD-08-E	7.09±0.07^{b}	10.70±0.00^{b}	20.15±0.01^{b}	22.71±0.03^{b}	27.53±0.00^{b}	33.95±0.04^{b}
16	NGB-01496	6.35±0.01^{ab}	11.36±0.01^{b}	19.36±0.40^{b}	22.88±0.03^{b}	25.18±0.00^{b}	34.32±0.02^{bc}

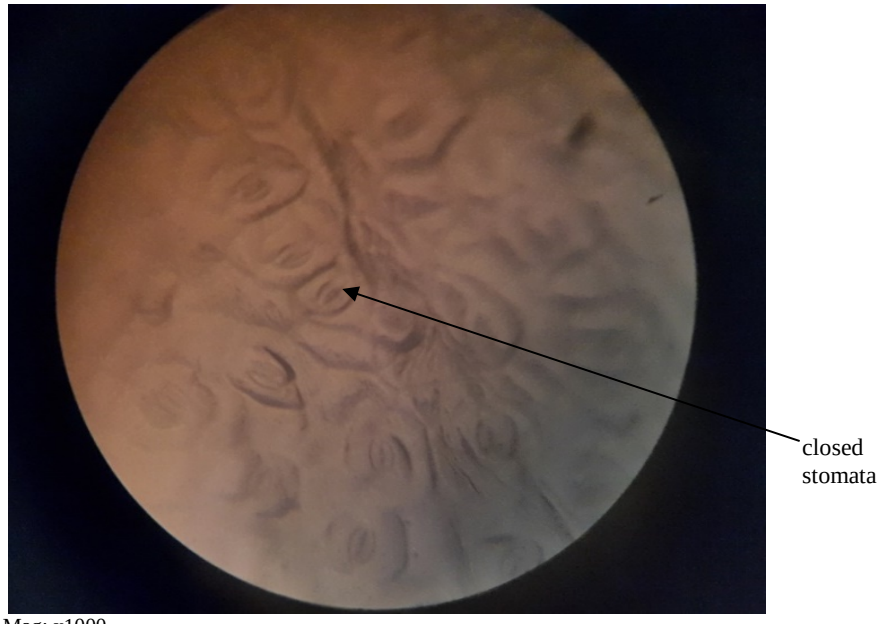
17	NGR-NI-27	5.06±0.00^{a}	12.07±0.02^{c}	17.19±0.06^{b}	22.65±0.01^{b}	22.94±0.07^{b}	35.41±0.01^{bc}
18	NGR-NI-25-A	10.90±0.02^{c}	11.87±0.01^{b}	22.67±0.01^{c}	22.77±0.02^{b}	33.76±0.02^{bc}	35.22±0.04^{bc}
19	NGR-PL-13	9.76±0.01^{b}	11.18±0.01^{b}	22.75±0.01^{c}	22.56±0.02^{b}	33.37±0.01^{bc}	34.40±0.03^{bc}
20	NGR-NS-15	4.23±0.00^{a}	11.55±0.01^{b}	12.86±0.01^{ab}	22.81±0.01^{b}	16.80±0.01^{a}	34.63±0.01^{bc}
21	NGR-NI-20-H	6.77±0.00^{ab}	10.20±0.01^{b}	19.35±0.03^{b}	21.12±0.18^{b}	26.33±0.02^{b}	37.41±0.04^{c}
22	NGB-01646-B	6.90±0.02^{ab}	11.25±0.02^{b}	19.61±0.01^{b}	22.46±0.18^{b}	26.54±0.01^{b}	34.58±0.01^{bc}
23	NGB-01646-C	9.15±0.01^{b}	11.38±0.01^{b}	23.94±0.01^{c}	22.56±0.03^{b}	29.47±0.00^{bc}	34.80±0.05^{bc}
24	NGR-NI-20-J	10.06±0.00^{c}	12.21±0.00^{c}	22.34±0.03^{c}	22.62±0.02^{b}	32.67±0.03^{bc}	35.64±0.01^{bc}
25	NGR-AD-27-B	10.25±0.02^{c}	10.37±0.02^{b}	22.98±0.03^{c}	23.57±0.05^{c}	33.70±0.26^{bc}	34.86±0.05^{bc}
26	NGR-JG-17-A	10.08±0.01^{c}	10.73±0.00^{b}	22.48±0.01^{c}	23.06±0.00^{c}	32.91±0.00^{bc}	34.12±0.01^{bc}
27	NGR-JG-17-B	7.05±0.00^{b}	11.89±0.00^{b}	18.97±0.03^{b}	22.59±0.21^{b}	27.07±0.03^{b}	35.29±0.02^{bc}
28	NGR-JG-17-C	9.08±0.00^{b}	11.63±0.01^{b}	21.49±0.04^{b}	22.94±0.01^{b}	31.17±0.05^{b}	34.81±0.01^{bc}

Values are means ± standard error, values followed by the same alphabet(s) in a column do not statistically differ at P>0.05 tested by Duncan Multiple Range Test. NST - Non stressed; ST - stressed.



Mag: x1000

Figure 2. Stomata opening of Bambara Groundnut in non-stressed condition



Mag: x1000

Figure 3. Stomata opening of Bambara Groundnut in stressed plant.

Discussion

Relative water content is the amount of water present in the leaf tissues. The significant differences observed in the relative water content of the bambara groundnut accessions in both stressed and non-stressed plants is an indication of genetic variability in the accessions ability to either absorb water from the soil or to control water loss through the stomata. It was observed that accession NGB-01491 had the highest relative water content both in the stressed and non-water stressed condition while accession NGR-KG-02 had the lowest relative water content. For some of the accessions studied the relative water content was higher in the non-stressed plants and others were higher in the stressed plants. High relative water content under drought stressed conditions was observed in all the accessions which helped to maintain water balance. Higher relative water content in leaves has been reported as selection criteria to breed plants tolerant to drought stress [RAHAMAN & al. 2000].

Assessment of pigment content has also become an effective means of monitoring plant growth and estimating photosynthetic productivity [CHEN & al. 2007]. The high chlorophyll content observed in the bambara groundnut accessions both in the stressed and non-stressed plants although higher in the non-stressed plants is an indication that the chloroplast was not destroyed by the water stress. The values observed were lower than that observed by VURAYAI & al. (2011) who recorded no significant differences in chlorophyll content between stressed and non-stressed plants suggesting that chlorophyll content in bambara groundnut landraces was not sensitive to water stress. FAROOQ & al. (2009) reported that chlorophyll content decreased in water stressed plants. Similar reduction in chlorophyll content in water stressed plants has also been reported in barley [ANJUM & al. 2003] and sunflower [KIANI & al. 2007].

The closed stomata observed in the water stressed accessions is an indication that bambara groundnut landraces responded to drought through stomata closure (Figure 3). This helped to reduce transpiration loss which is a characteristic of drought avoidance and escape mechanism. Stomata closure has been previously reported by COLLINSON & al. (1997) as a component of bambara groundnut drought resistance mechanism, however BLUM (2005) argued that stomata closure is a negative response to water stress in that it reduces carbon dioxide availability leading to yield reduction under water stress. Reduction in intracellular carbon dioxide, due to stomata closure resulted in reduced substrate availability for photosynthesis, hence leading to reduced yield in stressed plants.

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IMPACT OF PORTLAND CEMENT ON GROWTH OF BEAN CROPS

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Abstract: Cement manufacturing industries are responsible for environmental degradation at regional and global level. This study was undertaken the effect of port land cement on three different legume crops in pot. The cement treatment differently influenced on germination and biomass of all studied bean crops. Statistically, the root length of *Vigna radiata* was decreased at 0.50 g cement level. The cement treatment at similar level significantly reduced shoot and seedling length of *V. mungo*. The total seedling dry weight of *V. unguiculata* was significantly affected with cement treatment at 1.0 g. The sprinkled treatment at 2 g reduced tolerance index in seedlings of *V. unguiculata*, *V. mungo* and *V. radiata*, respectively.

Keywords: bean, cement pollution, pulse, seedling growth, tolerance, yield.

Introduction

The addition of organic and inorganic compounds in biosphere due to industrial activities produced damaging impact on plant growth. The alteration in the quality of air may leave profound effects on plant growth [JOSHI & al. 2009] and long exposure effect on lung function [MEO & al. 2017]. Cement is extensively used for construction and infrastructures all around the worldwide. The cement plants are a source of pollution [EKINCI & al. 2020]. Soil contamination by potentially toxic elements showed adverse environmental impacts [PALANSOORIYA & al. 2020] on living organism.

Cement has global impact on environment and is an important source of greenhouse gas emissions [SHEN & al. 2014] and vegetation [FAKHRY & MIGAHID, 2011]. The decrease in number of species near cement plant, stomatal clogging in *Inula grantioides* Boiss., decrease in leaf size for *Sida acuta* Burm f., and seedling height of *Datura innoxia* Mill. and chlorophyll contents of *Vigna unguiculata* (L.) Walp. due to cement pollution was recorded [SHAFIQ & IQBAL, 1987; ABDULLAH & IQBAL, 1991; AYANBAMIJI & OGUNDIPE, 2010]. The cement pollution also influence on plant growth, trace elements, chloroplast pigmentation, biomass production and nature, structure and composition of vegetation [FAKHRY & MIGHAHID, 2011; SHAFIQ & IQBAL, 2012; SHAFIQ & al. 2019].

Cowpea, black gram and mung bean are an important nutritional legume crops [KONGJAIMUN & al. 2013; IRITI & VARONI, 2017; KUMAR & al. 2017] and have the ability to grow in harsh environmental conditions [WIN & OO, 2016]. *Vigna mungo* (L.) Hepper is an annual herbaceous legume crop, cultivated for its edible seeds. Mash bean is widely cultivated throughout the tropics [EFLORA, 2018]. The ecologist are researching on the impact of environmental pollutants on plant growth. A significant increase of cement in the environment is resulting air pollution problem in Pakistan and affecting productivity of agricultural crops.

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The industrialization, rise of life quality of regions and strategies for economic prosperity leaded to an imminent increase of the quantity of eliminated waste [TARO & COMAN, 2020] and pollution [JOSHI & SWAMI, 2009]. Lucky Cement Factory was founded in 1996 [REUTERS, 2008]. Few studies have examined cement pollution impact on plant growth globally and less on this aspect on bean crop has been done in Pakistan. Therefore, in this experiment, the impact of Portland cement on growth of different bean crops was evaluated to find the level of toxicity and tolerance limit of bean to portland cement.

Material and methods

The sample of Portland cement was collected from lucky cement factory which is located near hub chowky, Blochistan, Pakistan. The certified seeds of bean crops (*Vigna unguiculata*, *Vigna mungo* and *Vigna radiata*) were purchased from the local super store and immersed for ½ an hour in distilled water to breakup seeds dormancy. The garden loam soil was passed through 2.0 mm sieve and was transferred in plastic pots having diameter 7.3 cm and 9.6 cm height. The filter paper was kept at bottom of pot having a small hole to minimize loss of chemical from soil. The ratio of garden soil was one part manure and two parts fine sand. Ten seeds were sown in each pot at 1 cm depth and irrigated with tap water with five replicates. Three best seedlings of same height were used for sprinkled treatment of cement at 0, 0.50, 1.0, 1.5, 2.0 g level on the aerial parts twice in a week. Height of plants were measured before sprinkling and pots positions were changed weekly to avoid any greenhouse effect. The experiment design was completely randomized for seven weeks. All the plants were removed from pots and washed with water for measurement of root, shoot and seedling length. The seedlings were dried in oven at 80 °C for 24 hours to record total plant dry weight.

The leaf area ratio, root/shoot ratio, leaf weight ratio and specific leaf area were described as given by REHMAN & IQBAL (2009).

An index of tolerance was determined by the following formula:

Mean root length in cement treatment / Mean root length in without cement treatment X 100.

Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) at $p<0.05$ was carried out on personnel computer using software packages SPSS version 10.

Results and discussion

Air pollution by cement has become a major threat to growth of plant [DEVARAJAN & al. 2018]. In present study there were variable response of cement pollution treatment on beans growth and tolerance indices were recorded (Table 1-3; Figures 1-2). The difference in seedling growth of bean were might be associated with increase in level of cement pollution treatment. Root growth responds incredibly dynamically in abio-stress conditions. The treatment at 0.5 g level significantly ($p < 0.05$) affected root growth of *Vigna radiata* (Table 1). The toxic effects are in agreement of other researchers' findings. The negative effects of O_3, SO_2 and NO_2 emissions from cement industry on seedling height of *Datura innoxia* Mill. and reduction in yield of cowpea plant were recorded also by different researchers [SALAMA & al. 2011; ADDO & al. 2013]. Plant response varies between species of a given genus for morphological characteristics. Based on the difference of morphological parameters from the control the order of tolerance to cement pollution 0.50 g cement treatment produced significant effect on shoot growth of all bean. Cement treatment of 1.5 g significantly decreased seedling length of *V. mungo* and at 2.0 g positively decreased seedling height of mung bean (Table 2). ZARGARI & SHOAR (2008) also reported similar results

regarding toxic effects of cement dust on growth characteristics performances of *Helianthus annuus* L. The soluble pollutants of cement dust were considered responsible for inhibition in germination of *Medicago sativa* [LAFRAGÜETA & al. 2014]. The treatment at 1.0 g level significantly affected total seedling dry weight of all bean crops. Similarly, decrease in phytomass of *V. mungo* due to exposure of cement kiln dust was investigated [PRASAD & INAMDAR, 1990]. PANDEY & KUMAR (1996) also confirmed he impact of cement dust pollution on biomass, chlorophyll, nutrients and grain characteristics of wheat.

A reduction in biomass of conifers to cement was due to cchanges in content of nutrient [MANDRE & al. 1999]. Statistically the poor development in leaf area ($cm^2 g^{-1}$) of *V. radiata* and *V. unguiculata* to cement treatment was noted (Table 3). Similarly, impact of dust particles depends on the amount of the dust responsible for the development of toxicity potential recorded [ŁUKOWSKI & al. 2020].

Table 1. Seedling growth performance of *Vigna radiata* (L.) Wilczek in different levels (0, 0.5, 1.0, 1.5 and 2.0 g) of cement treatment.

Cement treatments (g)	Leaf area cm^2	Root / shoot ratio	Leaf weight ratio	Specific leaf area ($cm^2 g^{-1}$)	Leaf area ratio ($cm^2 g^{-1}$)
0	37.69b ± 1.27	0.40a ± 0.12	0.41a ± 0.02	468.91a ± 73.94	187.06a ± 17.53
0.5	35.70ab ± 3.92	0.26a ± 0.09	0.41a ± 0.02	433.38a ± 48.29	179.00a ± 18.15
1.0	32.29ab ± 2.48	0.39a ± 0.08	0.41a ± 0.05	332.16a ± 41.77	180.62a ± 23.30
1.5	32.23ab ± 3.07	0.35a ± 0.04	0.41a ± 0.03	452.76a ± 42.42	178.11a ± 24.72
2.0	27.46a ± 1.93	0.33a ± 0.03	0.41a ± 0.03	451.35a ± 88.07	179.05a ± 25.40

Symbol used: ± standard error = Statistical significance determined by analysis of variance. Number followed by the same letter in the same column are not significantly different, according to Duncan's Multiple range test at P<0.05.

Table 2. Seedling growth performance of *Vigna mungo* (L.) Hepper in different levels (0, 0.5, 1.0, 1.5 and 2.0 g) of cement treatment.

Cement treatments (g)	Leaf area cm^2	Root / shoot ratio	Leaf weight ratio	Specific leaf area ($cm^2 g^{-1}$)	Leaf area ratio ($cm^2 g^{-1}$)
0	18.65a ± 1.40	0.22a ± 0.12	0.39a ± 0.03	166.93a ± 29.13	62.58a ± 7.44
0.5	17.82a ± 1.95	0.29ab ± 0.09	0.40a ± 0.03	409.79ab ± 77.72	159.23b ± 22.09
1.0	17.13a ± 1.23	0.32ab ± 0.08	0.37a ± 0.05	437.33ab ± 166.06	138.21ab ± 29.87
1.5	13.99a ± 1.76	0.23a ± 0.04	0.29a ± 0.07	537.78b ± 94.44	148.72ab ± 46.15
2.0	13.62a ± 1.33	0.45b ± 0.03	0.40a ± 0.01	530.64b ± 77.60	210.19b ± 28.28

Symbol used: ± standard error = Statistical significance determined by analysis of variance. Number followed by the same letter in the same column are not significantly different, according to Duncan's Multiple range test at P<0.05.

Table 3. Seedling growth performance of *Vigna unguiculata* (L.) Walp. in different levels (0, 0.5, 1.0, 1.5 and 2.0 g) of cement treatment.

Cement treatments (g)	Leaf area cm^2	Root / shoot ratio	Leaf weight ratio	Specific leaf area ($cm^2 g^{-1}$)	Leaf area ratio ($cm^2 g^{-1}$)
0	73.43b ± 5.04	1.19a ± 0.10	0.38ab ± 0.01	361.61b ± 40.34	140.90a ± 14.97
0.5	64.52ab ± 1.52	1.07a ± 0.07	0.42ab ± 0.01	240.65a ± 24.24	102.84a ± 6.69
1.0	58.74ab ± 9.10	1.14a ± 0.11	0.43ab ± 0.01	242.97a ± 40.19	104.47a ± 15.47
1.5	52.63a ± 4.06	1.44a ± 0.40	0.45b ± 0.02	232.25a ± 30.19	105.10a ± 14.12
2.0	61.03a ± 7.01	0.97a ± 0.20	0.36a ± 0.03	298.52ab ±39.54	103.54a ± 18.56

Symbol used: ± standard error = Statistical significance determined by analysis of variance. Number followed by the same letter in the same column are not significantly different, according to Duncan's Multiple range test at P<0.05.

The seedlings of beans were tested for tolerance to cement (Figure 2). The studies showed selective sensitivity to cement treatment exposure among plant species. 0.50 g cement treatment showed high percentage of tolerance in *V. radiata*, *V. mungo* and *V. unguiculata* seedlings. The lowest percentage of tolerance for *V. mungo* (64.70%), *V. radiata* (69.39%) and *V. unguiculata* (90.99%) was recorded with 2.0 g cement.

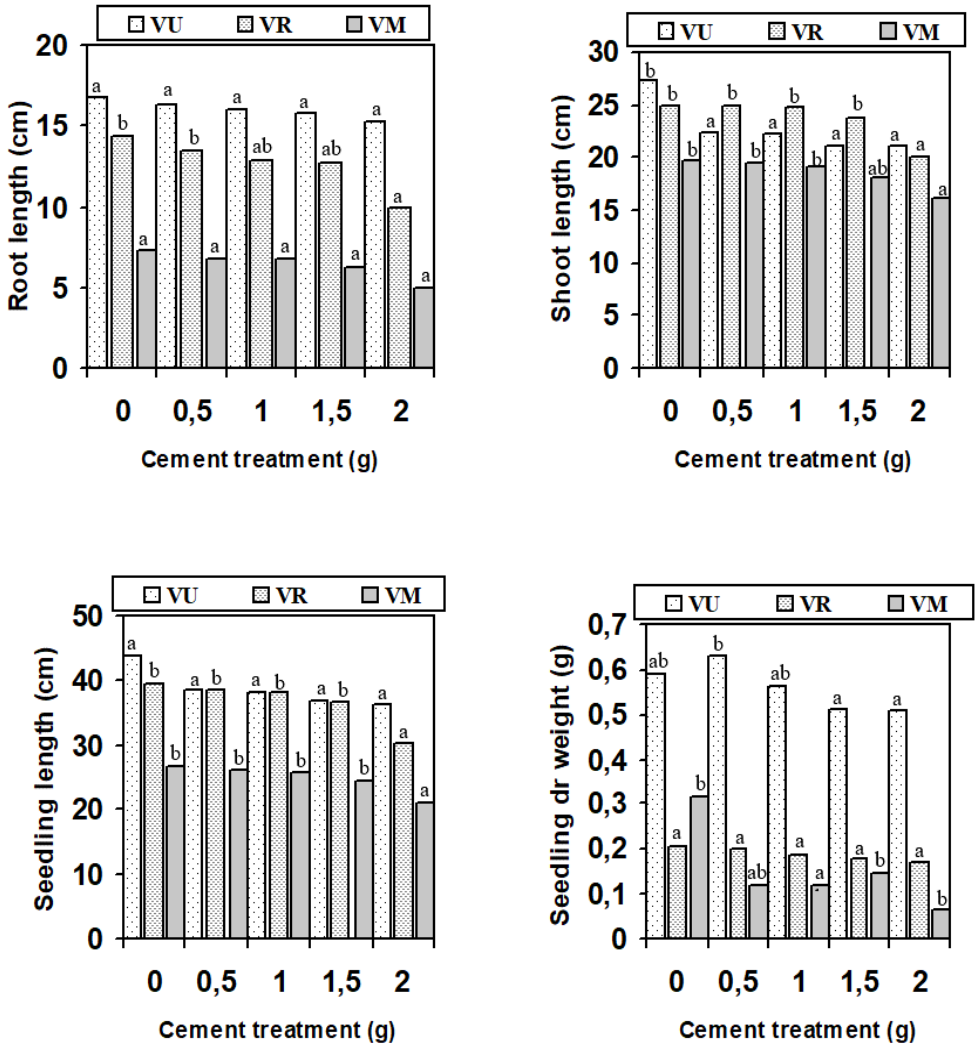

Figure 1. Effects of different level of cement treatment at 0, 0.5, 1, 1.5 and 2 g on root, shoot, seedling length and seedling dry weight (g) of VU = *Vigna unguiculata*, VR = *Vigna radiata*, VM = *Vigna mungo*. Values followed by the same letters on same bar chart are not significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.

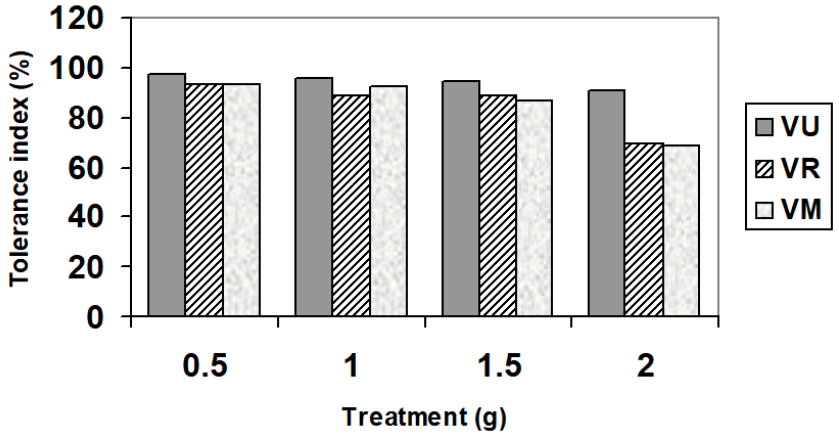

Figure 2. Tolerance indices of VU = *Vigna unguiculata*, VR = *Vigna radiata*, VM = *Vigna mungo*, against different level of cement treatment at 0.50, 1.0, 1.50 and 2.0 g.

Conclusion

The sprinkled treatment of Portland cement produced variable Portland cement toxic impact on seedling growth of bean crops. The response of bean plant to cement pollution varied between species of a genus. Plants also do not behave similar to pollutants and depends on level of tolerance and adaptation. The seedlings of *V. unguiculata*, *V. radiata* and *V. mungo* showed low percentage of tolerance to cement treatment at 2.0 g.

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EFFECTS OF AGE AT TRANSPLANTING ON MORPHOLOGICAL AND YIELD ATTRIBUTES OF THREE GENOTYPES OF SCARLET EGGPLANT

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Abstract: Three genotypes of scarlet eggplant obtained from National Agency for Conservation of Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria, were evaluated for the effects of transplanting ages on morphological and yield attributes at the Department of Plant Biology experimental field, Federal University of Technology, Minna, Nigeria. The experiment was set up using a randomized complete block design (RCBD) with four replicates. Standard procedures were followed when transplanting young seedlings of the various scarlet eggplant genotypes at week 3, 4, 5 and 6 after planting. The morphological parameters measured were plant height at transplanting, at maturity and at harvest, as well as number of branches and leaves at maturity. The yield parameters measured were number of fruits per plant, weight of fruits per plant, number of seeds per fruit and weight of 100 seeds per fruit for each of the week at transplanting for the selected eggplant genotypes. It was observed from the result that plants transplanted at week 3 had the highest plant height at maturity while plants transplanted at week 6 had the highest plant height in NHGB/09/128. However, plants transplanted at week 4 had significantly highest yield parameters ($p \leq 0.05$), i.e. Weight of fruit (2.44 g), Number of seeds/fruit (115.60), and Weight of 100 seeds (0.28 g). In NHGB/09/138, plants transplanted at week 4 had significantly highest plant height at maturity, plant height at harvest, number of branches per plant at maturity and number of leaves per plant at maturity (30.35 cm, 43.00 cm, 18.00 and 36.50 respectively). Similar trend was observed in weight of fruit (2.22 g) and number of seeds/fruit (98.20). In NHGB/09/132, plants transplanted at week 3 was significantly highest for all the yield parameters such as weight of fruit (1.15 g), number of seeds/fruit (89.80), and weight of 100 seeds (0.48 g). Thus, in all the three genotypes, plants transplanted at various weeks performed better than those not transplanted (control). It is therefore recommended that these ages at transplant be considered to be the best time for transplanting of these eggplant genotypes.

Keywords: genotypes, morphological parameters, scarlet eggplant, transplant, yield parameters.

Introduction

Scarlet Eggplant (*Solanum aethiopicum* L.) belongs to the family Solanaceae, [SUNSERI & al. 2010]. It is believed to have originated from Africa and have been domesticated from the wild relative *Solanum anguivi* Lam. [KUBIE, 2013]**.** Scarlet Eggplant is the most important solanaceous fruit crop after tomato (*Solanum lycopersicum*) [FRODIN, 2004]. Scarlet Eggplant is a species of nightshade grown for its edible fruit [CHEN & al. 2001]. The plant is a vegetable with an increasing popularity all over the world [PESSARAKLI & DRIS, 2003]. Itis also known by other names, such as the bitter tomato, Ethiopian eggplant and Ethiopian nightshade [USDA, 2015].The fruit should be harvested for consumption at a physiological non-mature stage when the seeds are still soft

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but are already developed [GAJEWSKI & ARASIMOWICS, 2004]. Unattractive attributes in eggplant such as bitter flavour, opaque skin, and floppy flesh is related to over matured fruit of the plant [MAYNARD, 1987].

Although excessive rainfall affects both vegetative growth and flower formation, the plant is well adapted to both wet and dry season cultivation. It requires optimum day temperature of 25°C-30°C and an optimum night temperature of 20°C-27°C [NORMAN, 1992; OBENG-OFORI & al. 2007]. A well-drained soil rich in organic manure and pH ranging from 5.5 to 6.5 is suitable for its production [RICE & al.1993].

Nutritionally, eggplant contains 92.5% water, 1% protein, 0.3% fat and 6% carbohydrate. It contains 30-50% Iron, Fibre, Potassium, Copper, Manganese and Vitamins which include Vitamins B1, B6, Folate and Niacin [HORNA & al. 2007; OKON & al. 2010; CHINEDU & al. 2011]. Medicinally, the eggplant when prepared is used in the condiments and products which is used in treating different diseases and health problems [BURKILL, 1985]. A meal of eggplant has been shown to be important to patients suffering from heart disease and raised intraocular pressure (glaucoma) [HARISH& al. 2008]. It contains nasunin, a potent antioxidant and free radical scavenger that have been proven to protect brain cell membrane fats [SABO & DIA, 2009]. It is also known for tackling malnutrition in Africa especially amongst children less than 5 years and women of child bearing age [CHADHA & OLUOCH, 2003]. As food, eggplants are cooked and used in the preparation of sauces for yam porridge and cocoyam especially the bitter species like *Solanum melongena* [ONWUKA, 2005].

In spite of all the vast economic importance, yet it is faced with some production challenges such as disease and pests. The use of transplanting (a cultural method) in the growing of eggplant would be of importance as the effect of transplanting on other related plants like pepper increased yield [CHARLES, 1998]. Transplanting is an economically and environmentally ideal alternative to seedling. Transplanting can potentially increase yield and quality, while decreasing costs, inputs and environmental impacts [VAVRINA & ARMBESTER, 2004]. Transplanting grants a greater flexibility in avoiding weather fronts since plants need less time in the ground. Transplants, once transferred to the field grow stronger than field seeded plants because of the sturdy root system that has been developed [CHARLES, 1998]. Transplanting offers great savings in seeds cost because the quantity used in the transplanting method is significantly lower than in direct seedling [CHARLES, 1998]. The use of cultural practices like transplanting of pepper [VAVRINA & ARMBESTER, 2004], tomatoes [CHARLES, 1998] has proven effective over time in terms of good quality and increase yield of pepper and tomatoes.

Materials and methods

Three genotypes of *Solanum aethiopicum* were obtained from National Agency for Conservation of Genetic Resources and Biotechnology (NACGRAB), Ibadan. The genotypes are NHGB/09/128, NHGB/09/132 and NHGB/09/138. Plastic flower pots each of 15 cm^3 capacity and 40 cm diameter which were used to nurse the plants were labeled with the code of each genotype collected. Twenty four (24) planting bags (each of 10 cm^3 capacity and 20 cm diameter) were labeled according to the code of each genotype into which the corresponding genotypes from the plastic flower pots were transplanted.

The experimental design was a Randomized Complete Block Design (RCBD) with 4 replications. The ages of transplant were taken from every other week from week three

after planting. The sandy loamy soil was scoped, mixed with water to loosen it and then mixed with Cow dung. The Cow dung was mixed thoroughly until it became homogenized with the soil. The mixing was done every week for four weeks. The planting bags were filled with the mixed soil, labeled and lined a day before transplanting.

Seedlings were raised on well prepared nursery beds; after sowing, the seeds were watered periodically. The beds were mulched using sticks to provide shade so as to protect the seedlings from harsh weather conditions. Watering was carried out every other day depending on the climatic conditions. Watering, hand picking of weeds and stirring of the soil to enhance aeration were carried out regularly. Seedlings were transplanted 3 weeks, 4 weeks, 5 weeks and 6 weeks after planting. Transplanting was done early in the morning to avoid transplant shock with four replicates from each genotype.

All the morphological parameters investigated were accessed using the standard procedures of AKINYELE & OSEKITA (2006) as well as DAUDU & al. (2015). These parameters include: plant heights at transplanting, plant heights at maturity, plant heights at harvest, number of branches at maturity, number of leaves at maturity, number of fruits/plant at harvest, weight of fruits at harvest, number of seeds/fruit and weight of 100 seeds per week. Quantitative data obtained were pooled for analysis. Analysis of variance (ANOVA) was used to compare the various mean values. Duncan Multiple Range Test (DMRT) post hoc test was used to separate the means. All values were considered significant at $P<0.05$.

Results and discussion

The result on the morphological characteristics of genotype NHGB/09/128 showedthat there is a significant difference ($P<0.05$) in plant height from different weeks. Meanwhile, the highest plant height at transplanting which is 7.38 cm produced by week 6 is significantly the same with week 5 but significantly different from every other week. The result on the yield of genotype NHGB/09/128 showed that the highest yield in terms of weight of fruit among the weeks is 2.44 g from week 5 and is significantly the same with week 3 while the lowest yield recorded in terms of weight of fruit is 1.33 g from week 5 which is statistically the same with week 6 (Table 1 and 2).

VAVRINA & ARMBESTER (2004) established the need to get appropriate time in transplanting solanaceous crops to enhance optimum yield production, especially pepper. In NHGB/09/132, the analysis of variance of the result showed that there is significant difference ($P<0.05$) among the weeks in terms of height. The highest height recorded is 7.35 cm which was produced by week 5 and is significantly the same with week 3, while the lowest plant height (3.25 cm) produced by week 4 is significantly different from all other values. The result on the yield of genotype NHGB/09/132 showed that the highest yield in terms of weight of 100 seeds among the weeks is 0.24 g from week 3 and is significantly different from every other week, while the lowest yield recorded is 0.22 from week 5 and week 6 which are significantly the same (Table 1 and 2).

In NHGB/09/138, the analysis of variance of the result showed that there is significant difference ($P<0.05$) among the weeks in term of plant height. However, the highest plant height at transplanting which is 8.55 cm produced by week 5 is significantly different from every other week. The highest yield in terms of weight of fruit among the weeks is 2.22 g from week 4 and is significantly different from every other week while the

lowest yield recorded in terms of weight of fruit is 1.02 g from week 6 which is significantly the same with week 5 (Table 1 and 2).

The result obtained in terms of morphological and yield parameter of scarlet eggplant (*Solanum aethopicum*) in genotypes NHGB/09/138, NHGB/09/132 and NHGB/09/128 indicated that they were affected by various ages at transplanting. For genotype NHGB/09/128, the height at maturity with number of leaves and branches did better at week 5 and week 6. This result is contradictory to LOU & al. (1993) who indicated that younger eggplant grew more vigorously after transplanting and yielded greater than older seedlings in China. The yield of the same genotype at week 4 had the highest yield in terms of weight of fruit per plant, number of seeds per fruit and weight of 100 seeds. This agrees with the work of SHUKLA & al. (2011, 2013) who reported that middle aged transplant produced more fruit than the younger or older transplants. This may be as a result of younger seedlings having less storage of food needed for vegetative growth and older transplants too mature, which might have limited vegetative growth, thereby producing non-vigorous plants having low yield and poor quality seeds. The older seedlings might have almost reached their flowering stage before been transplanted, this will definitely hinder higher fruit or heavier fruit production. Similar assertion had been made by SHUKLA & al. (2013). However, this result is in contrary to that of HOTTA & al. (1993) in Japan who determined that eggplant seedlings transplanted at 40 day after sowing were the most successful in summer trials which might be due to difference in planting periods and different genetic makeup of the plants. For genotype NHGB/09/132, the morphological parameters yielded better in week 3 and the result is in agreement with LOU & al. (1993) who indicated that younger eggplant grew more vigorously after transplanting and yielded greater than older seedlings in China.

The yield of genotype NHGB/09/132, in term of weight of fruit per plant, number of seeds per fruit and weight of 100 seeds, contradicts HARMON & al. (1991) who indicated week 6 and 7 eggplant grew more vigorously after transplanting. For genotype NHGB/09/138, the morphological parameters did better at week 4 which is also in contrary to the findings of HOTTA & al. (1993) who opined that transplant at week 40 days grew more vigorously than other weeks and the yield also did better in week 4. This result contradicts HARMON & al. (1991) who opined that older weeks did better that the earlier weeks. For all the genotypes in terms of plant yield, week 3 and week 4 transplants tend to have the highest degree of yield; similar report has also been reported by AKINROTIMI & ANIEKWE (2018) in green pepper.

Table 1. Agro-morphological parameters of the eggplant genotypes at different week intervals.

Age of plants	Heights (cm)			Morphology at maturity	
	Transplanting	Maturity	Harvest	Branches	Leaves
NHGB/09/128					
WEEK 3	5.48 ± 0.23^{b}	15.90 ± 0.65^{b}	29.00 ±1.00^{a}	8.50 ± 0.50^{a}	17.00 ± 4.50^{a}
WEEK 4	4.78 ± 0.23^{b}	14.75 ± 3.00^{b}	38.00 ± 1.00^{b}	12.00 ± 1.00^{a}	26.75 ± 2.75^{a}
WEEK 5	6.85 ± 0.10^{c}	15.00 ± 0.00^{b}	50.50 ± 1.50^{c}	14.50 ± 0.00^{a}	27.00 ± 0.00^{a}
WEEK 6	7.38 ± 0.63^{c}	11.00 ±1.00^{ab}	58.00 ± 3.00^{d}	12.00 ± 0.00^{a}	18.75 ± 0.75^{a}
CONTROL	0.00 ± 0.00^{a}	8.50 ± 0.50^{a}	31.00 ± 1.00^{a}	11.50 ± 0.50^{a}	12.00 ± 0.00^{a}
NHGB/09/132					
WEEK 3	3.70 ± 0.25^{b}	12.33 ± 1.33^{a}	27.38 ± 0.88^{a}	12.08 ± 1.58^{b}	31.25 ± 1.25^{b}
WEEK 4	3.25 ± 0.75^{b}	25.13 ± 5.63^{b}	31.25 ± 1.25^{a}	20.00 ± 0.50^{c}	61.75 ± 8.75^{c}
WEEK 5	7.35 ± 0.50^{c}	14.75 ± 0.75^{a}	38.25 ± 0.75^{b}	14.25 ± 1.25^{b}	40.25 ± 0.75^{b}

WEEK 6	6.70 ± 1.30 [c]	13.00 ± 1.00 [a]	43.25 ± 1.75 [c]	6.25 ± 0.25 [a]	15.00 ± 1.00 [a]
CONTROL	0.00 ± 0.00 [a]	12.00 ± 0.00 [a]	29.00 ± 1.00 [a]	5.00 ± 1.00 [a]	10.50 ± 0.50 [a]
Total	4.20 ± 0.91	15.44 ± 1.87	33.83 ± 2.04	11.52 ± 1.86	31.75 ± 6.30
NHGB/09/138					
WEEK 3	5.30 ± 0.20 [b]	16.63 ± 0.13 [a]	37.50 ± 1.00 [b]	8.50 ± 0.50 [ab]	13.50 ± 1.00 [a]
WEEK 4	6.00 ± 0.20 [bc]	30.35 ± 1.65 [b]	43.00 ± 1.00 [c]	18.00 ± 1.50 [c]	36.50 ± 4.00 [b]
WEEK 5	8.55 ± 0.50 [d]	17.00 ± 2.00 [a]	39.00 ± 1.00 [b]	11.75 ± 2.25 [b]	16.00 ± 1.00 [a]
WEEK 6	6.65 ± 0.15 [c]	14.50 ± 0.50 [a]	29.00 ± 1.00 [a]	8.50 ± 1.50 [ab]	12.25 ± 0.25 [a]
CONTROL	0.00 ± 0.00 [a]	13.50 ± 0.50 [a]	41.00 ± 3.00 [bc]	6.00 ± 0.00 [a]	11.00 ± 1.00 [a]
Total	5.30 ± 0.96	18.40 ± 2.08	37.90 ± 1.69	10.55 ± 1.46	17.85 ± 3.22

Values are means ± standard error, values followed by the same superscript on the same column is not significantly different at P>0.05 tested by DMRT.

Table 2. Yield parameters of the eggplant genotypes at different week intervals.

Yield	Weight of fruit (g)	Number of seeds/fruit	Weight of 100 seeds
	NHGB/09/128		
WEEK 3	2.34 ± 0.17[b]	100.80 ± 5.09[ab]	0.22 ± 0.00[a]
WEEK 4	2.44 ± 0.26[b]	115.60 ± 6.84[b]	0.28 ± 0.00[c]
WEEK 5	1.69 ± 0.28[a]	111.40 ± 8.34[b]	0.22 ± 0.00[b]
WEEK 6	1.33 ± 0.07[a]	89.20 ± 2.48[a]	0.23 ± 0.00[d]
CONTROL	1.21 ± 0.10[a]	88.15 ± 1.38[a]	0.22 ± 0.01[a]
Total	1.95 ± 0.14	104.25 ± 3.65	0.24 ± 0.01
	NHGB/09/132		
WEEK 3	1.15 ± 0.08[b]	89.80 ± 6.03[b]	0.48 ± 0.01[d]
WEEK 4	1.07 ± 0.05[b]	78.80 ± 3.75[ab]	0.46 ± 0.01[c]
WEEK 5	1.02 ± 0.09[b]	62.60 ± 7.05[a]	0.44 ± 0.00[b]
WEEK 6	0.94 ± 0.04[b]	69.80 ± 6.07[a]	0.44 ± 0.00[b]
CONTROL	0.62 ± 0.03[a]	60.44 ± 3.25[a]	0.34 ±0.00[a]
Total	0.96 ± 0.04	75.25 ± 3.56	0.46 ± 0.00
	NHGB/09/138		
WEEK 3	1.53 ± 0.10[b]	83.80 ± 2.71 [ab]	0.23 ± 0.00 [a]
WEEK 4	2.22 ± 0.04 [c]	98.20 ± 5.27 [b]	0.22 ± 0.01 [a]
WEEK 5	1.10 ± 0.08 [a]	64.20 ± 11.91[a]	0.22 ± 0.00 [a]
WEEK 6	1.02 ± 0.15 [a]	96.60 ± 3.12 [b]	0.30 ± 0.00 [b]
CONTROL	0.99 ± 0.15[a]	62.10 ± 6.68[a]	0.20 ± 0.10[a]
Total	1.47 ± 0.12	85.70 ± 4.42	0.24 ± 0.01

Values are means ± standard error, values followed by the same superscript on the same column is not significantly different at P>0.05 tested by DMRT.

Conclusion

It is therefore concluded that different age at transplanting tends to enhance variations in certain morphological and yield parameters in selected scarlet eggplant genotypes in Nigeria. It was also observed that transplanting at week 3 after planting is best for optimum yield in NHGB/09/132 whereas, transplanting at week 4 after planting is the best for optimum yield attributes in NHGB/09/128 and NHGB/09/138.

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INFLUENCE OF WATER HYACINTH (*EICHHORNIA CRASSIPES* (MART.) SOLMS) AND COWDUNG ON SOIL CHEMICAL PROPERTIES AND GROWTH OF OKRA (*ABELMOSCHUS ESCULENTUS* (L.) MOENCH)

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Abstract: This study evaluated the effect of Cow dung and Water Hyacinth (*Eichhornia crassipes*) on soil chemical properties, growth and yield of Okra (*Abelmoschus esculentus*). The study was conducted at the Department of Biological Sciences Garden of Usmanu Danfodiyo University, Sokoto in 2012/2013 wet season. The treatments consisted of cow dung (CD) and water hyacinth (WH) each at 0, 5, 10, 20 and 40% concentration incorporated with the top soil. The experiment was arranged in Randomized Complete Block Design (RCBD), replicated five times. Soil chemical analysis for pH, %Org. M, %Org. C, %N, P, K, Na, Ca, Mg and C.E.C. was carried out before and after planting using standard methods of soil analysis, chemical content of CD and WH were determined. The results shows that both CD and WH enhanced soil chemical contents and at 40% application of CD and WH organic matter, N, P and C.E.C. of the soil. Numbers of leaves, leaf length, number of fruits, fruit dry weight, stalk dry weight and root length were taken for ten weeks, 2 weeks after planting (2WAP). The result revealed that plant height and numbers of leaves of okra were significantly ($p<0.05$) affected with CD and WH. The application of CD and WH at 5% enhanced growth and yield of okra, as well as the soil nutrients status. Application of CD increased plant height and numbers of leaves in Okra more than WH this suggest their potential in ameliorating soil nutrient status and growth of okra.

Keywords: cow dung, growth, mineralization, okra, water hyacinth.

Introduction

Tropical soils are adversely affected by low soil fertility and erosion, causing deterioration of nutrient status and changes in soil organism populations (Economic Commission of Africa, 2001). Use of inorganic fertilizers can improve crop yield and soil pH, total nutrient content and nutrient availability, but it's limited due to scarcity, high cost, nutrient imbalance and soil acidity. Therefore, use of organic manure as a means of maintaining and increasing soil fertility has been advocated [RODALE, 1984; ALASIRI & OGUNKEYE, 1999; SMIL, 2000]. Animal manures, when efficiently and effectively used could ensure sustainable crop productivity by immobilizing nutrient that is susceptible to leaching. Nutrients contain in manure are released more slowly and stored for longer time in the soil ensuring longer residual effects to improved root development and higher crop yields [SHARMA & MITTRA, 1991; ABOU EL-MAGD & al. 2005]. Manures are usually

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applied at high rate; they give residual effect on the growth and yield of succeeding crops [MAKINDE & AYOOLA, 2008]. Improvements of environmental conditions as well as the need to reduce cost of fertilizing crops are reason for advocating the use of organic materials [BAYU & al. 2006] organic manure improve soil fertility by activating soil microbial biomass [AYUSO & al. 1996]. Application of manure sustains cropping system through better nutrient recycling [EL-SHAKWEER & al. 1998]. Manures serve as a source of macro and micro- nutrients in available forms, thereby improving the physical and biological properties of the soil [ABOU EL-MAGD & al. 2006].

Soil fertility over the years have been achieved using traditional resources of farmyard manure and crop residues in composted forms as well as inorganic fertilizer [SRIDHAR & ADEOYE, 2003]. These however have their attendant constraints in the procurement and application of organic and inorganic manure to improves soil fertility, such as high cost (chemical fertilizers) and relatively low nutrient value (crop residues). One largely overlooked resource available for soil fertility remediation is the use of non-traditional or traditional organic materials such as *Eichhornia crassipes* which is a common weed and cow dung as an animal waste in the tropic that are generally considered as environmental nuisance in land and rivers where they exist.

Water hyacinth is one of the fastest growing plants knows and reproduces primarily by runners and stolons, which eventually form daughter plant. It produces large quantities of seeds and which could be viable up to thirty years. The common water hyacinth *Eichhornia crassipes* is vigorous grower known to double their population in two weeks [BARRETH, 1980]. It is also found in lotic water and they are probably brought into such aquatic environment by water current [NDIMELE, 2012]. They are hard, very difficult to eradicate because they can survive in extremely harsh conditions. During unfavorably condition such as drought, the plant sinks into the water and remains dormant until the condition of the environment becomes favorable for growth. Water hyacinth grows well in nutrient rich water but do not tolerate brackish water because of its salinity level [SOOKNAH & WILKIE, 2004]. The conducive temperature and seasonally or constantly low salinity of River Niger has supported the proliferation of water hyacinth. Coupled with this and its high rate of reproduction has made it a serious threat to the continued use of the affected Nigeria water resources. The rate of aquatic invasion by water hyacinth in Nigeria water bodies is alarming. It forms a thick mat covering rice paddles, blocking canals and channel, impeding navigation, halting fishing, sweeping away building for mosquito [CHUKWUKA & UKA, 2007; NDIMELE & al. 2011]. It caused problem to the dependents of Jebba riverine community of River Niger, who use the river for their socio-economic activity. It impedes water transportation by preventing access to the source of their livelihood and reduces or prevent accessibility to fishing ground especially during the rainy season which is the period when fishing activities is intense and more profitable. This poses a great hindrance to the socio-economic potential of this water if appropriate and effective control is not introduced. This study compares the effects of water hyacinth and cow dung as manure on soil chemical properties and growth performances of okra.

Materials and methods

The research was conducted in the Biological Sciences Garden of Usmanu Danfodiyo University, Sokoto (which is situated between longitude 5°11′30″ E and 5°14′30″ E and latitude 13°8′30″ N and 13°7′0″ N of Wamako Local Government Area of

Sokoto State [MAISHANU & al. 2017]. The research started on 15th June, 2012 and ended on 29th August, 2012.

Water hyacinth sample were collected from river Niger, down hydro-power station dam in Jebba, which is in the border between Kwara and Niger state on latitude 9°10′ to 9° 55′ N and longitudes 4°30′ E to 5°00′ E [ADENIJI, 1991]. The samples were collected on 4th March, 2012, air-dried for 6 weeks and pulverized by grinding. The cow dung was collected from cattle ranch close to the postgraduate hostel of Usmanu Danfodiyo University, Sokoto. Seeds of *Abelmoschus esculentus* Ex-Sokoto (a local variety from vegetable growers at Kwalkwalawa, Village Sokoto), were collected from Sokoto central market.

Proximate and mineral analysis of Water Hyacinth and cow dung were carried out according to methods described by PIRIE (1955). The following parameters were determined: Moisture content, Ash content, crude fibres, Crude lipid, Nitrogen and Crude protein, Carbohydrate, Phosphorous, Sodium, Potassium, Calcium, Magnesium and Nitrogen. Soil pH, Carbon, Potassium, Calcium Magnesium and Nitrogen before and after planting were determined following methods described by URIYO & SINGH (1974).

Experiment design and treatment

The experiment was arranged in a randomized complete block design with 10 treatments in 5 replications. The treatments consisted of 5 concentrations (0, 5, 10, 20, and 40%) each of residual form of Water hyacinth, and cow dung. Each concentration was incorporated into 10 kg of garden soil. The experiment was conducted in the polythene bags which were all perforated for aeration and placed in open space of Biological Sciences. Both the water hyacinth and cow dung manure were applied to the soil 7days before planting. Data on plant height, number of leaves, leaf length and root length, were taken in two weeks' interval, while fruits per plant were also counted. Dry matter on fruits and stalk were taken after drying in an oven at 120 °C for 72 hours.

Data analysis

The data obtained from soil and okra plant were subjected to statistical analysis of variance using the mixed model procedure in SPSS Version 20, and Significant means were separated using least significant difference (LSD). Mean value was used to compare the baseline proximate composition and mineral contents of cow dung and Water hyacinth.

Results and discussion

Chemical analysis of cow dung and Water Hyacinth

Result of proximate and mineral analysis of cow dung and water hyacinth is presented in Table 1. Cow dung higher % Ash and % Fibre compared to Water Hyacinth. Conversely, Water Hyacinth had significantly higher % moisture, % Lipid, % Carbohydrate, Calcium and Magnesium than Cow dung. However, there were no significant differences between cow dung and Water Hyacinth in their % Nitrogen, % Crude protein, Phosphorus, Sodium and Potassium. The content of calcium and magnesium content was 47.65 and 6.84 mg/kg respectively in water hyacinth while their values in cow dung were comparatively low.

Effect of cow dung and Water Hyacinth on chemical properties of soil before and after planting

The result of soil analysis prior to planting and after planting is presented in Table 2. There was no significant difference in soil pH, before and after amendment with cow dung and water hyacinth. Different combinations (Concentrations) of Cow dung and Water Hyacinth had significant effects on soil pH. There was no significant interaction between manures and concentration in pH value. The organic matter content of 71.30% was obtained in the soil prior to planting and significant ($p<0.001$) difference was observed between the treatments, with highest value of 1.71% in water hyacinth amended soil and 0.72% in cow dung. The same trend was observed in varying the concentration of the treatments with highest value of 2.16% recorded from 5% concentration and 20% concentration gave the least value of 0.70%. There was significant ($p<0.01$) difference in interaction between manures and concentration in the values of organic matter. Percentage carbon prior to planting was 0.75%, but significant ($p<0.001$) difference was observed between the treatments, with highest value of 1.07% in water hyacinth and cow dung had the least value of 0.42. The percentage organic carbon was significantly ($p<0.01$) affected by concentration, highest value of 1.25% in 5% concentration and the least value of 0.40% organic carbon was obtained from 20% concentration. There was a significant ($p<0.01$) interaction between the manures and concentration in the content of organic carbon. The percentage nitrogen prior to amendment was 0.05%, but significant ($p<0.001$) difference was observed between the treatments, with highest value of 0.47% in cow dung and least value of 0.25% in water hyacinth, similar trend was also observed in varying the concentration of the treatment with 0.84% recorded from 40% concentration and 0.11% from control soil. There was no significant effect in interaction between manures and concentration in percentage nitrogen.

Phosphorus content of the soil prior to planting was 0.22 mg/kg, while significant ($p<0.001$) difference was observed between the treatments with 4.10 mg/kg in cow dung as the highest value and water hyacinth had 3.08 mg/kg. The same trend was observed in concentration of the treatments with highest value of 4.33 mg/kg recorded from 40% concentration and lowest value of 2.26 (mg/kg) from the control soil. There was significant ($p<0.001$) difference in interaction between manures and concentration in phosphorous content.

The calcium content of the soil prior to planting was 0.37 cmol/kg, but there was significant ($p<0.05$) difference between the treatments and water hyacinth had the highest with 0.67 cmol/kg and lowest of 0.53 cmol/kg was recorded in cow dung. However, there was no significant effect of concentration on the calcium content. Manure and concentration interaction effect on calcium content was not significant. Magnesium content of the soil prior to planting was 0.83 cmol/kg. There was no significant effect between cow dung and water hyacinth, and concentrations of treatments on magnesium content. However, interaction effect was significant with 0.22 cmol/kg of potassium prior to planting. The manures, their concentrations, and interaction had no significant effect on potassium content of the soil. Initial content of sodium prior to planting was 0.63 cmol/kg. Cow dung treatment had significantly higher sodium content 0.70 cmol/kg than water hyacinth 0.35 cmol/kg. Although concentration had no significant ($p>0.05$) effect on the values of sodium the interaction effect was significant. Finally, 2.14 cmol/kg was observed prior to planting for the cation exchange capacity (C.E.C.) of the soil. Soil treated with cow dung had significantly higher ($p<0.001$) C.E.C. value of 4.53 cmol/kg than soil amended with water hyacinth 2.17 cmol/kg.

There was a significant ($P < 0.001$) effect of varying concentration on the C.E.C. value with 4.41 cmol/kg in 5% concentration and 2.63 cmol/kg from the control soil. There is significant interaction between the manure and concentration on soil C.E.C.

Effect of cow dung and Water hyacinth on growth and yield attributes of okra plant

Results in Table 3 showed the height of Okra grown in soils amended with cow dung and water hyacinth manure and showed a significant ($p<0.05$) from the 6^{th} to the 10^{th} week after planting with plants grown in cow dung amended soils being consistently taller. However, variation between the treatment's concentrations were not significant. Table 4 shows the mean effect of manure and their concentrations on number of leaves. The result obtained at week two and six, showed there were no significant ($p>0.05$) effect of the treatments, whereas the effect was significant ($p<0.05$) in week four with cow dung given the highest mean value of 8.0 followed by the water hyacinth with mean value of 7.4 cm. At week eight there was significant ($p<0.01$) difference, where the cow dung recorded highest mean value of 8.32 cm and the water hyacinth gave 5.68 cm. At week ten there was a significant ($p<0.01$) effect in numbers of leaves, the cow dung recorded the highest mean value of 6.80 cm, while the least value of 5.04cm was recorded from the water hyacinth. However, there was no significant effect ($p>0.05$) on the numbers of leaves throughout the period of the trial. Similarly, leaf length was not affected significantly ($p>0.05$) by manure and concentration (Table 5) as well as root length, dry matter and numbers of fruits (Table 6) throughout the period of the experiment.

Table 1.Baseline mean values for proximate analysis of cow dung and Water Hyacinth.

Parameters	Cow dung	Water hyacinth
% Moisture	8.2	9.17
%Ash	47	14.17
%Lipid	1.5	10.17
%Fibre	12	2.70
%Nitrogen	0.94	0.62
%Crude protein	5.88	3.85
%Carbohydrate	33.07	69.15
Phosphorous(mg/kg)	98.59	97.50
Sodium(mg/kg)	0.81	1.11
Potassium(mg/kg)	1.89	2.85
Calcium(cmol/kg)	0.35	47.65
Magnesium(mg/kg)	0.6	6.84

Table 2. The effect of cow dung and Water Hyacinth on soil chemical parameters.

Treatment	Parameters									
	pH	%Org.M	%Org.C	%N	P(mg/kg)	cmol/Kg Ca	Mg	K	Na	CEC
Before Planting	6.67	1.30	0.75	0.05	0.22	0.37	0.83	0.22	0.63	2.41
Manure:										
CD	6.51	0.72	0.42	0.47	4.10	0.53	0.99	0.28	0.70	4.53
WH	6.59	1.71	1.07	0. 25	3.08	0.67	1.04	0.25	0.35	2.17
SE±	0.05	0.91	0.11	0.03	0.06	0.05	0.06	0.02	0.07	0.07
LSD	Ns	0.000^{***}	0.000^{***}	0.000^{***}	0.000^{***}	0.004^{*}	Ns	Ns	0.000^{***}	0.000^{***}
Concentration (%)CD/WH										
5	6.41	2.16	1.25	0.14	3.71	0.58	1.12	0.26	0.57	4.41
10	6.53	0.99	0.57	0.24	3.77	0.58	1.07	0.29	0.46	3.14
20	6.61	0.70	0.40	0.46	3.50	0.62	1.02	0.22	0.35	3.65
40	6.68	1.22	0.71	0.84	4.33	0.62	0.95	0.33	0.54	2.90
0	6.54	1.00	0.77	0.11	2.62	0.62	0.92	0.25	0.70	2.63
SE±	0.08	0.30	0.17	0.05	0.10	0.08	0.09	0.04	0.11	0.11
LSD	Ns	0.003^{**}	0.008^{**}	Ns	0.000^{***}	Ns	0.007^{*}	Ns	0.013^{*}	0.000^{***}

*, **, *** Denote Significant at 5, 1 and 0.1 Percent Probability Level, Respectively. Ns Denote effect not Significant. CD = cow dung. WH = water hyacinth, CEC = Cation Exchange Capacity.

Table 3. Effect of cow dung and Water Hyacinth on plant height in Okra.

Treatment	WAP				
	2	4	6	8	10
Manure					
Cow dung	7.61	18.92	32.10	55.70	55.9
Water Hyacinth	7.33	17.66	27.00	44.90	44.9
SE±	0.627	1.257	2.33	4.14	4.18
LSD	Ns	Ns	0.036^{*}	0.013^{*}	0.013^{*}
Concentration (%) CD/WH					
0	7.58	20.08	25.6	50.4	51.4
5	7.02	16.21	30.5	51.0	51.2
10	6.36	15.94	28.6	47.7	47.8
20	8.48	19.27	31.1	49.8	49.7
40	7.92	19.95	31.9	52.7	52.0
SE±	0.99	1.99	3.69	6.55	6.61
LSD	Ns	Ns	Ns	Ns	Ns

*, Denote Significant at 5, Percent Probability, Ns Denote effect not significant at % 5 probability level

Table 4. Effect of cow dung and Water Hyacinth on the number of leaves.

Treatment	WAP				
	2	4	6	8	10
Manure					
Cow dung	4.20	8.32	8.52	8.32	6.80
Water Hyacinth	4.28	7.4	8.04	5.68	5.04
SE±	0.34	0.54	0.58	0.71	0.58
LSD	Ns	0.024*	Ns	0.001***	0.005**
Concentration (%) CD/WH					
0	4.70	8.20	7.00	6.90	5.00
5	4.60	8.00	8.30	7.50	6.10
10	4.00	6.50	7.50	5.90	5.20
20	4.10	8.40	9.70	7.80	6.90
40	3.80	7.30	8.20	6.90	6.40
SE±	1.09	0.86	0.92	1.12	0.92
LSD	Ns	Ns	Ns	Ns	Ns

*, **, *** Denote Significant at 5, 1 and 0.1 Percent Probability Level, respectively.
Ns Denote effect not Significant.

Table 5. Effect of cow dung and Water Hyacinth on the leaf length.

Treatment	WAP				
	2	4	6	8	10
Manure					
Cow dung	3.56	10.14	10.05	9.98	9.23
Water Hyacinth	3.08	9.11	10.86	9.30	8.42
SE±	0.27	0.66	0.68	0.63	0.63
LSD	Ns	Ns	Ns	Ns	Ns
Concentration (%) CD/WH					
0	3.63	10.06	9.69	8.96	8.20
5	3.46	10.25	10.91	10.67	9.17
10	3.20	9.12	10.00	8.68	8.19
20	3.40	10.00	11.34	10.68	9.76
40	2.92	8.69	10.35	9.23	8.79
SE±	0.43	1.05	1.08	1.00	1.00
LSD	Ns	Ns	Ns	Ns	Ns

Ns = no significant effect at 5% probability level. WAP = Weeks after planting, SE = Standard error

Table 6. Effect of cow dung and Water Hyacinth on the number of fruits, root length and dry matter: (Stalk Dry Weight and Fruit Dry Weight).

Treatment	WAP			
	NoF	RL (cm)	StlkDW	FrtDW
Manure				
Cowdung	2.08	15.63	4.71	33.6
Water Hyacinth	1.80	12.92	5.67	30.3
SED±	0.45	1.97	0.64	2.96
LSD	Ns	Ns	Ns	Ns
Concentration (%) CD/WH				
0%	1.60	11.60	5.28	32.1
5%	2.20	13.37	3.39	34.4
10%	1.80	13.37	3.29	34.4
20%	2.20	15.87	6.00	30.9
40%	1.90	17.96	5.99	28.7
SE±	0.78	3.12	1.01	4.69
LSD	Ns	Ns	Ns	Ns

ns = no significant effect at 5% probability level.
NoF = Number of fruits, RL = root length, StkDW = Stalk dry weight, FrtDW = Fruit dry weight.

Discussion

Proximate and mineral analysis of both cow dung and water hyacinth used in this study showed that they varied in their nutrient composition. The low moisture content of water hyacinth observed may be as result of presence of cellulose, hemicellulose and pectin that are likely higher in cow dung, and are insoluble in water, which is in line with the findings of NUKA & DUBEY (2011), who reported the response of water hyacinth manure contribute to the nitrogen availability released during mineralization on growth and yield of *Brassica juncea*. The high ash content observed in cow dung may be as result of nature of plants on which the animals feed, and is contrary to the findings of NUKA & DUBEY (2011), who reported the response of water hyacinth manure on growth attribute of *Brassica juncea* with low ash content. High lipid content was observed in water hyacinth followed by cow dung. This may be attributed to the high level of lignin, present in water hyacinth which is oily in nature, this goes contrary to the finding of NGULDE & al. (2018), who reported higher amount of lipids in cow dung. The fibre content of cow dung was higher than, that of water hyacinths this may be as a result of types and nature of plant materials the animals feed on. There was no significant difference in the percentage nitrogen of cow dung and water hyacinth, though value obtained from cow dung was slightly higher compared to waters hyacinth, the low content of nitrogen in water hyacinth may be as a result of incomplete mineralization of in the two manure. This is also in line with the reports of MAKINDE & al. (2011), who reported effect of mineral fertilizer and organic manure on growth, nutrient content and yield of Cassava plant and observed complete mineralization in inorganic fertilizer while incomplete mineralization took place inorganic manure. However, percentage carbohydrate in water hyacinth was higher than that of cow dung. This may be attributed to fact that the plant has the ability to synthesis their own carbon compound while the Cow that produces the dung depend on the plants for their carbon compound. This is contrary to the finding of NUKA & DUBEY (2011) who investigate the effect of water hyacinth manure on growth of India Mustard plant and reported low amount of carbon compound from the water hyacinth. The phosphorous

content of cow dung was slightly higher than that of water hyacinth, sodium, potassium does not show much difference. Similar trend was reported by NUKA & DUBEY (2011) and MAKINDE & AYOOLA (2012). However, the calcium content of water hyacinth was very high compared to that of cow dung; this is also in line with the findings of MAKINDE & AYOOLA (2012) who reported the growth and yield of okra using cow dung and poultry manure. High content of magnesium was found in water hyacinth and cow dung, the magnesium content of water hyacinth was more than that of cow dung this also corroborates with the findings of NUKA & DUBEY (2011), they recorded high amount of magnesium in water hyacinth compared to cow dung.

The soil analysis before planting showed that the soil pH was slightly acidic, however, at post-harvest 40% concentration gave the highest value of pH, while the least value was recorded from 5% concentration. The slightly acidic condition may be due to the fact that Sokoto State is in semi-arid region, and one of the characteristics of soil of this region is its neutral to slightly acidic nature, which is in agreement with the finding of SHUKRY & al. (2007), who recorded high amount of pH in calcareous soil on growth vigour, water relations, mineral uptake and content of fatty acid and protein of Flax plant. High amount of organic matter was recorded from soil amended with water hyacinth followed by control soil. This may be as result of mineralization process, the manures might have undergone. This is in line with the finding of GUNNARSSON & PETERSON (2007), who reported high amount of organic matter and fast mineralization of water hyacinth in agriculture land and energy production. Highest value was obtained from soil treated with 5% concentration and soil treated with 20% concentration gave the least value in terms of interaction between the manure and concentration. This is in conformity with the studies of SHARMA & MITTRA (1991), on the effect of different rates of application of organic manure. Highest percentage carbon was obtained from soil treated with 5% water hyacinth, and the least value was obtained from soil treated with 20% cow dung. This could be attributed to the high amount of organic carbon earlier recorded from the water hyacinth been added to the okra in addition to the synthesis one above the ground. While the low content of carbon in soil treated with cow dung may be as result of dependence of animals on plant materials for carbon compound. This is contrary to the finding of NUKA & DUBEY (2011), who reported high amount of organic carbon in soil amended with water hyacinth. This is in line with the finding of OKWUAGWU & al. (2003), who confirm effect of organic and inorganic manure on soil properties and yield of okra. Soil treated with cow dung gave the highest value of nitrogen, and the least value was observed in control soil. This may be as result of actions of digestives enzymes that might have acted on plant material eaten by the animals before being used as organic manure, which may in turn hasten the mineralization process of cow dung. This is in line with the finding of ZAMAN & al. (2017), who reported gradual increase of soil nitrogen amended with cow dung at varying concentration. However, there was significant effect in nitrogen concentration, with 40% concentration showing the highest value, while the least value was recorded from the control soil. This may be as result of increment in the application of manures and is in conformity with the finding of WIDJAJANTO & al. (2001) who conduct studies on the release of nitrogen from water hyacinth incorporated in to soil-crop system. Soil treated with cow dung recorded the highest value of phosphorous while the control soil gave the least value. The high content of phosphorus, in soil treated with cow dung may be attributed to addition of other molecules or ion from the animal excretal or other biomolecules, while the least value showed by the control soil may be due to the fact that phosphorous is the

most limiting element in the soil. This could be supported by the result of CHUKWUKA & OMOTAYO (2008), who reported the effect of *Tithonia* green manure and water hyacinth compost application on nutrient depleted soil and also similar to the finding of WIDJAJANTO & al. (2001). However, varying the concentration, 40% concentration had the highest phosphorous content, while the lowest was recorded from the control soil. This could be as a result of enzymes activities that might have taken place on the manures, decomposing them to reasonable extent and is supported by CHUKWUKA & OMOTAYO (2008). There was interactive effect in phosphorous contents of manures and concentration. The Soil amended with water hyacinth contained high amount of calcium followed by control soil. This may be attributed to the fact that water hyacinth might have added part of its calcium content to the soil. This is in accordance with the findings of OJENIYI & al. (2013), on effect of organic manure on soil physico-chemical properties, nutrient uptake and yield of cocoyam plant. There was no effect was observed in varying the concentration of the manures. The interaction between the manures and concentration show no effect. This is in line with the finding of CHUKWUKA & OMOTAYO (2008). There was no favorable effect in magnesium and potassium, but soil treated with water hyacinth gave the highest values while the least value was recorded from control soils. Similarly, there was no significant effect in magnesium and potassium content, in terms of concentration. But significant effect of interaction was observed in magnesium. However, the potassium content does not show any interaction effect. This was supported by the finding of OJENIYI & al. (2013), who reported the effect of poultry manure on soil physical properties and nutrient uptake of cocoyam. The sodium content of soil treated with cow dung had the highest value while the least value was obtained from the soil amended with water hyacinth. This may be attributed to availability of more Na^+ in cattle, than the plant material. There was no significant effect in concentration, but effect was observed in interaction between the manures and concentration. This is contrary to the finding of AKANDE & al. (2010), who reported the response of okra to organic and inorganic fertilizer. The value of CEC recorded from cow dung and least value from water hyacinth, this explains the potentials of cow dung in supplying adequate basic cations as nutrient source than the water hyacinth. This corroborates with the report of OKWUAGWU & al. (2003), who reported the effect of organic and inorganic manure on soil properties and yield of okra. In varying the concentration of manures, high amount of CEC was recorded from soil treated with 5% concentration, while the least value was obtained from control soil. Same trend was observed in interaction between the manures and concentration,

There was promising increase in pant height due to the application of organic manure, but the application of cow dung showed more promising increase in plant height compared to water hyacinth, this may be as a result of interaction between the organic manure and growth hormones in the various part of the plant, as reported by ABUSALEHA & SHAMUGAVETU (1988), using animal manure with inorganic fertilizer as reported by YADAV & al. (2004). It is also possible that improved growth observed in Okra with the application of organic amendments may be due to the presence of high phosphorous content in both manure which increased the availability of native soil phosphorous and increased biological activity of microbes and a similar opinion had been expressed by SINGH & SRIVASTAVA (1970). Moreover, there was an appreciable effect on the number of leaves for both treatments. Although cow dung showed more advantageous effect over water hyacinth, which could be supported by the study of SOLOMON & al. (2012), who reported the effect of cow dung and N.P.K. fertilizer on the growth of maize. Other parameters like

leaf length, number of fruit/plants, fruit dry weight/plant, stalk dry weight/plant as well as the root length showed no significant effect as a result of the treatments application.

Growth parameters, such as plant height, number of leaves/plant, leaf length/plant, number of fruits, root length/plant, fruit dry weight and stalk dry weight all do not show positive effect even upon varying the concentration of cow dung or water hyacinth from 5-40%. This is in line with the study of SEYEDBAGHERI (1999), who conducted a study on effect of organic fertilizer on the vegetative growth of plants even at maturity stage, high dosage application is recommended from the study.

The cow dung and Water hyacinth do not show any interactive effect on growth and yield parameters, however, cow dung showed favorable effect on plant height and number of leaves. This may be as a result of interaction between the cow dung and growth hormone, more also, plant treated with cow dung might have gotten access to high amount of growth hormone than those treated with water hyacinth. This result is in agreement with that of OLUBUNMI & al. (2011), who reported an increase in the yield and nutrients uptake in two vegetable plant using poultry manure and contrary to their work at maturity stage, using organic manure and mineral fertilizer, that both organic and in organic fertilizer are effective in vegetative growth of crop and yield.

Conclusion

The result of this study shows that the application of Cow dung and water hyacinth at different concentration positively influenced some of the parameter studied. Cow dung and water hyacinth application have been found to increase organic matter, organic carbon, N, P, Ca, Na and CEC of the soil, their application at different concentration also suggest the soil with higher concentration of cow dung or water hyacinth has positive influence on organic matter, organic carbon, P, Mg, Na, and CEC of the soil and exert positive influence in the vegetative growth and yield of okra plant. However, cow dung and water hyacinth could be applied in high dose for improved soil fertility and growth and yield of okra.

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RESPONSE OF *MDR1* AND *PDR1* ATP-BINDING CASSETTE-TYPE TRANSPORTER GENES TO BIOTIC SIGNALS IN WHEAT FLAG LEAF TIPS

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Abstract: ATP-binding cassette-type (ABC) transporters are highly implicated in detoxification processes but not restricted to detoxification processes. Several ABC transporters including wheat Lr34 were shown to function in plant defense responses and secretion of plant antimicrobial compounds. Members of multidrug resistance (MDR) proteins and pleiotropic drug resistance (PDR) proteins were studied in wheat. MDR1 and PDR1 expression was relatively stable in all the developmental stages but responded differentially to salicylic acid and fumonisin B1. *In silico* analysis indicated that both MDR1 and PDR1 had expression levels in all analyzed parts of wheat.

Keywords: ABC-transporter, biotic stress, disease resistance, protein kinase, wheat Lr34.

Introduction

Plants must continuously defend themselves against attack from pathogens. Because their immobility precludes escape, plants possess both a preformed and an inducible defense capacity. Toxin detoxification represents one of the most effective defense mechanisms and one of the most significant findings was from the study wheat stripe rust resistance gene *Lr34* [KRATTINGER & al. 2011]. *Lr34* has been associated with both wheat leaf rust and stripe rust [SINGH, 1992]. It is a widely-studied benchmark for multiple fungal pathogen resistance and is controlled by a single ATP-binding cassette (ABC) transporter gene [KRATTINGER & al. 2011].

Confirmation of the hypothesized ABC transporter motif is a significant step in understanding disease resistance in wheat, as disease resistance protein in *Arabidopsis* homologs have been documented as having ABC transporter motifs [KANG & al. 2011]. First identified as transporter proteins located in eucaryotic cell membranes, ABC proteins have been found to be ubiquitous and their role in cellular metabolism to be broader than transporting molecules across the cell membrane. The common unifying characteristic of ABC proteins is a unique amino acid signature sequence. In addition, the ABC proteins are a constitution of subunits forming hetero- or homodimer. The subunits consist of a transmembrane domain (TMD) and nucleotide-binding domain (NDB). The number and general combination of subunits of ABC are varied but generally fall into nine subfamilies. ABC proteins in the subfamilies Pleotropic Drug Resistant (PDR), Multidrug Resistance (MDR) and Multidrug Resistance-associated Protein (MRP), have been documented as having a role in managing biotic and abiotic stresses in other organisms [SHANG & al. 2011; SASAKI & al. 2002; THEODOULOU & al. 2003].

LR34 is predominantly expressed in adult foliar tissues, particularly of the flag leaf, and the highest transcript levels were found in the leaf tip, corresponding to the tissues

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that exhibit the phenotypic difference between the tolerant and susceptible wheat lines [KRATTINGER & al. 2009]. Wheat varieties with functional *LR34* alleles can be distinguished phenotypically by the development of leaf tip necrosis in adult flag leaves [KRATTINGER & al. 2009; KANG & al. 2011]. Despite its resistance-conferring properties, *LR34* is not responsive to pathogen inoculation, suggesting that it has constitutive rather than induced functions [KANG & al. 2011]. Here, we report our study on differential expression of different ABC transporters in flag leaves of Fielder cultivar, which does not possess expression resistance genes when challenged with stripe rust [RANDHAWA & al. 2012]. We also studied the effect of transient expression of a wheat mitogen-activated protein kinase kinase on the expression of MDR and PDR. Our data suggest that the tested ABC proteins could be induced in a stripe rust-susceptible cultivar.

Material and methods

Plant growth

Triticum aestivum seeds of the Fielder variety were sterilized in a solution of 70% ethanol for 2 min, then transferred to a bleach solution of 25 ml of bleach, 25 ml of distilled water and 10 µl of triton extract. The seeds were then rinsed 10 times in distilled water. After drying 5-6 seeds were potted in autoclaved Pro-mix BX soil fertilized with 7-9 granular of slow release NPK fertilizer (14:14:14). The seeds were then placed in a growth chamber set for 16 hr at 22 ºC in the light and 8 hr at 18 °C in the dark. The seed were watered every second day.

Treatment with fumonisin B1 and salicylic acid

Wheat seeds were left to grow for a period of three weeks. The leaves were harvested and cut into segments of 1.5-2 cm and were treated 5µM fumonisin B1 (FB1, Sigma-Aldrich) and 100 µM salicylic acid (SA, Sigma-Aldrich) or ddH_2O as a control. The leaves were then placed in a vacuum infiltration chamber for a period of 30 minutes. The treated leaf segments are then placed upwards on top of a filter paper in a Petri dish. At intervals of 0 hr, 6 hr, 24 hr and 48 hr the leaf segments were collected and placed in Falcon tubes and snap frozen in liquid nitrogen. The materials were then stored in a -80 °C. Protein kinase inhibitors staurosporine and SB202190 were from Sigma-Aldrich Canada.

RNA extraction

Total RNA was extracted from wheat leaf tissues (100 mg) using TRIzol Reagent Kit (Invitrogen) according to manufacturer's protocol. After TRIzol extraction, deoxyribonuclease I kit (Invitrogen) was used to eliminate genomic DNA contamination in the sample, and the cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol. RT-PCR was carried out under the following conditions: 94 °C for 1 min; 1 min at 94 °C, 1 min at 61 °C, and 1 min at 72 °C for 25 cycles; and then 10 min at 72 °C.

RT-PCR

PCR primers for *Triticum aestivum* PDR-type ABC transporter (*PDR1*) and *Triticum aestivum* MDR-type ABC transporter (*MDR1*) were designed using NCBI's Primer-BLAST tool. RT-PCR was performed for *PDR1* (FJ185035.1) and *MDR1* (AB055077.1). Wheat *PDR1* primers were 5'-GACCGTAAGAGAGACGCTCG-3' (forward) and 5'-GCAGGAGG

GAGATCATCACG-3' (reverse). Wheat *MDR1* primers were 5'-GCAGAGAAAAGAGTGG TTACAAC-3' (forward) and 5'-AGGAATGTGCAAGGTAAAGTCAC-3' (reverse). Wheat actin was used to standardize the volume of cDNA loading per treatment. The following conditions were used for RT-PCR: 94 °C for 1 min, 60 ºC for 1 min, and 72 °C for 30 seconds for 28 cycles, and then 10 min at 72 °C.

Gene expression analysis with Genenvestigator

GenBank accession numbers AB055077.1 and FJ185035.1 were used for *Triticum aestivu*m *PDR1* and *MDR1*, respectively. From the Genevestigator databases, target sample probes, either from *T. aestivum* or a related cereal organism, were identified. The target probes were put through Condition Search in Genevestigator to identify sample experiments and expression levels in the anatomy.

Results and discussions

Effect of fumonasin B1 and salicylic acid on MDR1 and PDR1 expression

As shown in Figure 1, FB1 and SA up-regulated both genes relative to the treatment with water, the control. The expression patterns between the FB1 and SA treatment across both genes are generally similar (Figure 1). Compared to the control *MDR1* expression seemed to be sustained through to the 6 hrs inoculation period, thereafter the expression level decreased for both SA and FB1 treatment. FB1 and SA did not affect expression at 0 hrs across all treatments as indicated by similar band strengths as the control. *MDR1* expression was slightly more consistent when treated with FB1 over SA treatments. *PDR1* expression was observed when treated with either FB1 or SA, no expression was observed in the control at any time interval.

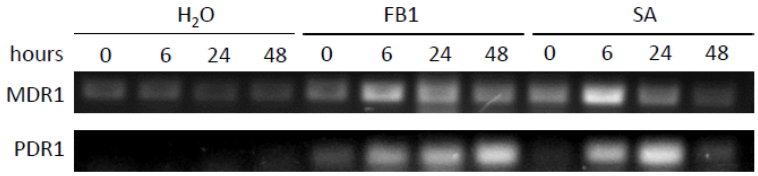

Figure 1. Effect of fumonasin B1 and salicylic acid on *MDR1* and *PDR1* expression. The effect was analysed by RT-PCR. Actin was used as internal standard. This experiment was performed three times with similar results.

Effect of protein kinase inhibitors on FB1- or SA-induced expression of *MDR1*and *PDR1*

To study the downstream signaling pathways FB1- or SA-induced *MDR1* and *PDR1* gene activation was examined in the presence of staurosporine, a broad spectrum protein kinase inhibitor, and SB 202190, an inhibitor specific to the p38 class MAPKs. Staurosporine reduced the FB1- or SA-induced expression of *MDR1* and *PDR1*, whereas inhibitors of p38 class MAPK had no effect (Figure 2).

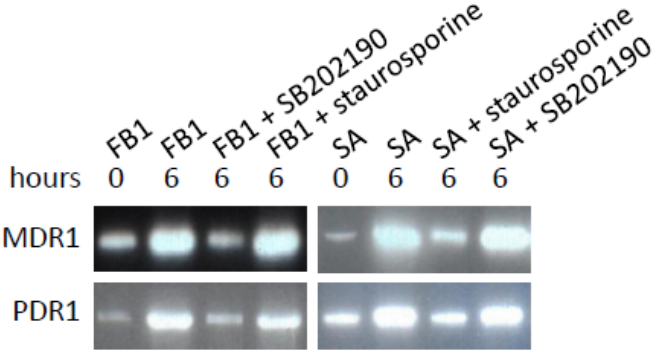

Figure 2. Effect of protein kinase inhibitors on FB1- or SA-induced expression of *MDR1* and *PDR1*. The effect was analysed by RT-PCR. Kinase inhibitors (kinase inhibitors at the concentration of 1 μM for staurosporine and 350 nM for SB 202190) were included in the treatment. Actin was used as an internal standard. This experiment was performed three times with similar results.

Expression analysis of *MDR1* and *PDR1* with Genenvestigator

In silico analysis indicated that both *MDR1* and *PDR1* had expression levels in all analyzed parts of wheat. The expression strength is equitably distributed across all parts of the plant as no part of the plant was statistically different from the other (Figure 3). There is no significant difference in expression in the various plant organs.

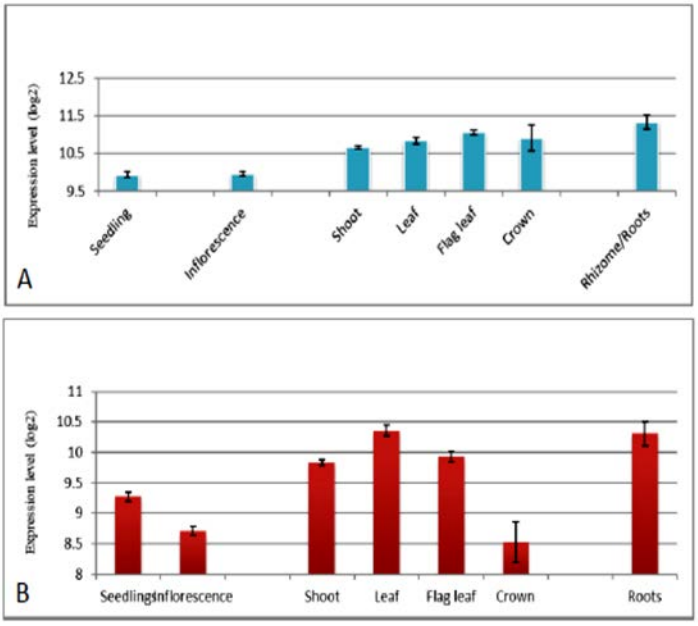

Figure 3. Expression levels across anatomical parts of the plant. A. *PDR1*, B. *MDR1*.

Discussion

The study of ABC transporters in plant disease resistance has been much enhanced with the confirmation of the presence of an ABC transporter motif within the Lr34/Yr18/Pm38 coding region [KRATTINGER & al. 2009]. Later on, Lr34 was also shown to function as a transporter of the ABCG subfamily [KRATTINGER & al. 2011]. A family of ABCG transporter genes in wheat cultivars was shown to be enhanced when wheat plants were challenged by a deoxynivalenol-producing *Fusarium graminearum* strain [MUHOVSKI & al. 2014]. Some ABC transporters such as Lr34 were shown to function in secretion of plant antimicrobial compounds [KRATTINGER & al. 2011; HWANG & al. 2016]. Here, members of multidrug resistance (MDR) proteins and pleiotropic drug resistance (PDR) genes in wheat were studied.

At time 0 hr *PDR1* levels are very low or non-existant. Leaf samples treated with H_2O showed no expression at any of the treatment time intervals. When treated with FB1 and SA *PDR1* expression was enhanced. The results appear to mirror previous research where members of the PDR family of ABC were expressed and up-regulated in the presence of biotic and abiotic stress [KANG & al. 2011]. PDR is localized on the plasma membrane [KANG & al. 2011], thus ideal for early detection of any pathogen. *MDR1* expression pattern was similar whereby the bands at all time intervals were more pronounced when treated with SA and FB1 in comparison to the control (Figure 1). In the control, bands of *MDR1* was observed, though faint, indicating there is a background level of MDR1 while the cell is in homeostasis. SA is known to be involved in plant defense [ZHANG & LI, 2019], thus the relevance of the nearly identical expression patterns of *PDR1* and *MDR1* between the SA and FB1 treatments may indicate that the proteins partially contributed to plant defense through the salicylic pathways.

Reversible phosphorylation plays a critical role in plant defense responses to pathogen attack [XING & LAROCHE, 2011]. Previously, components of mitogen-activated protein kinase (MAPK) cascades in wheat were shown to be part of the defense responses of wheat to leaf rust and *Fusarium* head blight infection [GAO & al. 2011, 2016]. The existence of multi-member families at each tier of the cascade is supported by recent genome-wide analysis [GOYAL & al. 2018; WANG & al. 2016]. To examine if MAP kinase pathways are involved in the activation of *PDR1* and *MDR1* by FB1 or SA, staurosporine and SB202190 were included the treatment. Our data seem to indicate an involvement of phosphorylation, but it may not be mediated through a p38 class of MAP kinase.

In silico analysis aided by Genevestigator suggests that with no biotic stresses both *MDR1* and *PDR1* had expression levels in all analyzed parts of a wheat plant. The expression strength is equitably distributed across different organs of the plant as no part of the plant was statistically different from the other (Figure 3). It should be noted that many ABCG/PDR-type ABC transporters are induced by biotic stresses. Among these are AtABCG40/AtPDR12 [CAMPBELL & al. 2003], AtABCG16 [JI & al. 2014], and potato StPDR1 to StPDR4 [RUOCCO & al. 2011]. A significant challenge is the fact that we still have not identified the substrate transported by Lr34. While our data further support the potential function of *MDR1* and *PDR1* in wheat defense responses, one of the main attempts should be the identification of the exact substrates of these transporters.

Notes on contributors

Kipkios TUBEI is a MSc student supported by a research grant from Bayer Crop Science Inc. Lucas CHURCH is an undergraduate student.

Tim XING is an associate professor and a plant molecular biologist with a special interest in cell signaling and plant-microbe interactions. He teaches plant physiology, molecular plant development, and cell signaling.

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MOLECULAR DIVERSITY IN SELECTED LANDRACES RESISTANCE TO BLAST PATHOGEN (*PYRICULARIA GRISEA* COOKE EX SACC.) OF PEARL MILLET (*PENNISETUM GLAUCUM* (L.) R. BR.) GERMPLASM FROM NORTHERN NIGERIA

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Abstract: Germplasm collection and sourcing for resistant genotypes among the available landraces through characterization and quantification of genetic diversity is essential for introgression in plant breeding programs. Therefore, study on molecular diversity of pearl millet (*Pennisetum glaucum*) landraces was carried out to characterize the crop accessions for resistance to blast pathogen as well as identify elite accession(s) for the crop improvement. Thirty five (35) pearl millet germplasm were collected from the major cultivated states in Nigeria and were screened for blast resistant genotypes on the field using a Randomized Complete Block Design (RCBD). Selected potentially resistant accessions for blast resistant were further confirmed under screen house condition and evaluated for genetic diversity using random amplified polymorphic DNA-PCR. Out of the 35 accessions screened for blast, 14 were potentially resistant to blast disease (with disease score of ≤ 3.9 on 1-9 scale). Further nursery screening of the potentially resistant accessions to blast showed that NS-YEL-02 was the most highly resistant followed by NG-ZB-01 with severity disease score of 0.00 and 0.33, respectively. Molecular diversity of selected 14 resistant and 2 susceptible accessions using random amplified polymorphic DNA showed no specific marker for resistant and susceptible accession to the disease. A total of fifty nine (59) amplified fragment bands with 10 DNA primers was generated, of which 53 (89.83%) were polymorphic and 6 (10.17%) were monomorphic. Genetic similarity among the accessions varied from 0.18 to 1.44 with an average gene diversity value of 0.74. Clustered dendrogram of the 16 accessions revealed two major cluster groups; two susceptible (KD-CK-01 and NGB501) accessions with similarity coefficient 1.14, and 14 resistant accessions. Clustering of the selected landraces base on resistance and susceptibility by RAPD techniques, indicate its possibility for indirect selection of blast resistant genotypes for the crop. The high resistance and clustering of NS-YEL-02 singly in a group by the makers indicate the uniqueness and its prospect for selection, as elite parent accessions in blast disease resistance breeding programs of pearl millet.

Keywords: blast pathogen, molecular diversity, pearl millet, RAPD, resistant.

Introduction

Genetic improvement of crop species mainly depends on the extent of variability for economically important traits present in the gene pool [SINGH & al. 2013]. Pearl millet, *Pennisetum glaucum* (L.) R. Br. – Family Poaceae is endowed with enormous genetic variability for various morphological traits, yield components, adaptation and quality traits which have accumulated over a long century [SHAH & al. 2012]. This huge variability had not been well exploited for its improvement, with the crop been attacked by many diseases

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such as blast, downy mildew, ergot, rust and smut. Among these, leaf blast caused by *Pyricularia grisea* had been identified as a major problem with a serious threat to pearl millet productivity [PAWAR & al. 2016]. In Northern Nigeria, where the crop is mainly produced, its production had been reported to be undermined and yield is usually uncertain due to leaf blast constraints [ABDULLAHI & al. 2006; SHARMA & al. 2013; AZARE & al. 2020]. However, pearl millet landraces had been reported to be rich sources of resistance to various biotic and abiotic stresses as well as traits for improving grain and fodder quality [KHAIRWAL & al. 2007]. This resistance advantage of the local landraces has been exploited only to a limited extent in a very unsystematic manner for the crop improvement in Nigeria [ANGARAWAI & al. 2016].

According to OBIDIEGWU & al. (2014), developing of superior, high yielding and biotic tolerance cultivars could be achieved through characterization and understanding of genetic variations available in the germplasm. The use of molecular markers for quantification of genetic diversity present within cultivars had been reported to be of great importance in improving inheritable disease resistance in crop through selection of efficient and diverse combination of parents [ABOU-TALEB & al. 2010]. Random Amplified Polymorphic DNA (RAPD) marker technique has been effectively used to detect genetic variation among variant resistant genotypes in different crop species such as groundnut genotypes to foliar diseases [MONDAL & al. 2005], wild and cultivated potato (*Solanum demissum*) variety to late blight [ABOU-TALEB & al. 2010], characterized resistant and susceptible pearl millet to downy mildew [MAHATMA & al. 2011] without prior sequence information. Therefore, collection of germplasm, characterization and quantification for genetic diversity among the selected resistant landraces was carried out using RAPD markers.

Material and methods

Collection of Germplasm

A total of thirty five (35) pearl millet accessions were collected from the major growing states (Adamawa, Gombe, Jigawa, Kano, Nasarawa, Niger, Kaduna, Sokoto, Taraba, and Zamfara) in the Northern Nigeria; these included twenty five (25) landraces from farmers through direct contact and 10 landraces from National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria.

Field screening for blast resistance

Field screening of the collected landraces was carried out for selection of potential resistant accessions to blast disease, using Randomized Complete Block Design (RCBD) with a total of 25 plants per plot and 75 plants per accession in 3 blocks. The evaluation was done by examining 10 randomly selected plants per plot for blast symptoms and the percentage disease incidence (PDI) was calculated LUBADDE & al. (2014). The leaf blast severity of each accession was scored on plot basis using a scale of 0-9 developed by IRRI and reviewed by THAKUR & al. (2011) (Table 1).

$$\text{PDI (\%)} = \left(\frac{Total\ Number\ of\ Disease\ Plant}{Total\ Number\ of\ Selected\ Plants\ Per\ Plot}\right) \times 100$$

Isolation, Identification and Pathogenicity of Blast Pathogen

Isolation and identification of blast pathogen was done using modified methods of THAKUR & al. (2009a) and MANANDHAR & al. (2016). Infected tissues (leaf and node) were obtained from blast susceptible plants in the field and culture on potato dextrose agar (PDA). Pure cultures of the isolate were prepared and identified base on culture morphology and microscopic characteristics of the spores and mycelia.

Pathogenicity of the identified fungi isolate (*P. grisea*) was carried by spray-inoculation of potted seedling (10 seedlings per pot) of susceptible accession (KD-JB-01) with aqueous conidial suspension (1×10^5 conidia/mL) at fifteenth (15) days after planting using mineral oil (glycerin) as carrier. The set-up was incubated at screen house temperature 27 ± 3 °C for 24 hour; after which the seedlings were exposed to relative humidity of above 90% under mist for 7 days. The experiment was replicated four times and monitored for the development of blast symptoms for re-isolation of the pathogen [SHARMA & al. 2013].

Table 1. Blast severity (0-9 grade) disease rating scale used for Pearl Millet.

Grade	Disease severity	Host response
0	No lesions	Highly Resistant
1	Small brown specks of pin point size	Resistant
2	Larger brown specks	Moderately resistant
3	Small, roundish to slightly elongated, necrotic spots, about 1-2mm in diameter with brown margin	Moderately resistant
4	Typical blast lesions usually confined to the area between main veins, covering < 5% of the leaf area	Moderately susceptible
5	Typical blast lesions covering 6-10% of the leaf area	Moderately susceptible
6	Typical blast lesions covering 11-25% of the leaf area	Susceptible
7	Typical blast lesions covering 26-50% of the leaf area	Susceptible
8	Typical blast lesions covering 51-75% of the leaf area and many leaves dead	Highly susceptible
9	Typical blast lesions covering 76% and above of the leaf area and all leaves dead	Highly susceptible

Source: THAKUR & al. (2011).

Nursery Screening for Blast Resistance

The selected potentially resistant accessions on the field were planted in experimental bags of 30 cm diameter (10 seeds/pot) filled with 15 liters of mixed sterilized sand-loamy soil and manure (3:1 weight/weight). The bags were labeled properly and arranged in a completely randomized design (CRD) with three replicate per accession in a nursery and maintained at temperature of 27 ± 3 °C in a screen house. The germinated seeds at 14 days-old; were spray-inoculated with an aqueous conidial suspension (1×10^5

conidia/mL) using mineral oil (glycerin) as carrier and covered with transparent polyethylene bags to prevent cross contamination. The seedlings were incubated at 27 ± 3 °C for 24 hour and after which polyethylene bags were removed; exposed to relative humidity of above 90% under mist for 7 days. The seedlings were monitored for development of blast symptoms and severity of the disease was scored and recorded on twenty (20) plants for each accession between 8 to 21 days after inoculation using a progressive scale of 0-9 developed by THAKUR & al. (2011) (Table 1).

Molecular evaluation

The molecular analysis was carried out at Bio-Science Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

DNA extraction

The DNA extraction from fresh young leaf (2 weeks old) of the 16 nursery screened pearl millet accessions was carried out using Cetyl Trimethyl Ammonium Bromide (CTAB) procedure as described by DAUDU & al. (2016) with slight modifications. One hundred milligram (100 mg) of the leaf sample (for each accession) was ground in liquid nitrogen to a fine powder and transferred into 2.0 ml Eppendorf tube. 700 µL of pre-heated DNA extraction buffer (100 mM tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB, and 1% 2–mercaptoethanol) was added and vortex thoroughly for 10 minutes to obtain even suspension. The samples were incubated at 65 °C for 30 minutes with intermittent shaking and cooled for 2 minutes. A volume of 750 µl of chloroform-isoamyl alcohol mixture (24:1) was added to each sample tubes and centrifuged at 12,000 rpm for 10 min (Sigma centrifuge model 4K15C) to obtain the protein. The separated aqueous and viscous supernatant was transferred to a freshly labelled eppendorf tube. Ice-cold isopropanol (500 µl) was added to an approximately 400 µl of the supernatant. The prepared samples were gently mixed and incubated in -20 °C for 1 hour to precipitate the nucleic acid (DNA). The samples were centrifuge at 12,000 rpm for 10 minutes and the supernatants were decanted to the last drop in a fume chamber leaving the DNA in the tubes. The DNA was washed in 70% ethanol and vacuum dried for 10 minutes. Degradation of the co-isolated RNA was achieved by re-suspending DNA pellet in 200 µl of TE buffer containing 3µl of RNase (40 mg/µl) mixed properly and incubated at room temperature for 30 minutes.

RNA was remove from each tube after incubation period, by adding Chloroform:isoamylalcohol (24:1) mixture and centrifuged at 12,000 rpm for 10 minutes. The aqueous layer containing the DNA was transferred to fresh tubes and 15 µl of 3M sodium acetate (pH 5.2) and 300µl (2 volume) of absolute ethanol were added; incubated at –20 °C for 30 minutes and centrifuged at 12,000 rpm for 10 minutes. The supernatant was decanted carefully, and 200 µl of 70% ethanol was added to the tubes; centrifuged at 12,000 rpm for 5 minute to obtain the DNA pellets by carefully decanting the supernatant from each tube and vacuum dried for 10 minutes. The quality of the DNA was checked on 0.8 % agarose gel electrophoresis and quantified with Nanodrop Spectrophotometer (ND-1000) before storage at -20 °C.

DNA amplification

Ten set of randomly amplified DNA primers were used for amplification. The conditions reported by WILLIAMS & al. (1990) for creating RAPD markers by PCR in pearl millet template DNA were used. PCR Amplification reaction was carried out in a 25µl reaction volume which contained 2.0 µl of DNA template, 2.5 µl of 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 2.0 µl of 2.5 mM dNTPs, 0.2 µl of Taq polymerase and

10 µl of RAPD primer in a thermocycler model 9600. The reaction was conducted in 40 cycles and each cycle consists of denaturation at 94 °C for 15 sec, followed by annealing at 37 °C for 40 sec and primer extension at 72 °C for 60 sec. Different levels of annealing temperatures (37, 42 and 72 °C) and number of cycles (45 and 55 cycles) were used for the amplification conditions. The products were then amplified on 2% agarose gels using 0.2 µg ml^{-1} ethidium bromide stains and ran at 80 Volts for 4 hrs. The result was visualized under UV light, estimated using 50 base pair (bp) standard size marker and the fragment band photographs were then taken.

Data analysis

The data obtained on disease incidence were expressed in percentage and that of severity were subjected to analysis of variance (ANOVA) to determine the level of significance within the means using SPSS software version 18. Analyses of the Molecular data were done using the maximum composite likelihood model. The analysis involved 16 nucleotide sequences. Each band in the RAPDs profile was taken as an independent locus with two alleles. The bands produced for the primer were scored one (1) for presence or zero (0) for absence manually and a binary matrix was generated for further analysis. Evolutionary analyses were conducted in MEGA6 [TAMURA & al. 2013].

Results

Field evaluation for blast infection

Field evaluation of the 35 accessions for blast resistance showed that there was significant different ($p < 0.05$) in level of resistance of the genotypes to blast infection. Fourteen accessions equivalent to 40.00% of the total accessions are potentially resistance to the blast infection with disease score ranged of 0.78 to 3.50. Accessions NS-GIN-03 (0.78), NS-YEL-02 (0.80) and NG-ZC-01 (0.96) were highly resistant (HR), NG-ZB-01 (1.50) and NG-ZA-08 (1.90) were resistant (R) and nine (9) of the accessions were moderately resistant (MR) (Table 2). A total of 60.00% of the accessions were susceptible to blast infection with highest susceptible accession being KD-JB-01 (7.70) and least moderately susceptible as NS-GAN-05 (4.33). In terms of potential disease incidence (PDI), KD-JB-01 was the most infected genotype with the value of 96.25% and NS-GIN-03 (11.25%) being the least.

Screen house evaluation for blast infection

The result of screen house evaluation of the fourteen selected potential resistance and two susceptible (KD-CK-01 and NGB501) accessions as check is presented in Table 3. Out of the fourteen selected potential resistance accessions, nine (9) of the accession proved to be blast resistance with NS-YEL-02 being the most resistance to the pathogen with disease severity score of 0.00 when compared to the field value of 0.80. This is followed by accession NS-YEL-07 (0.67) and the least resistance being NS-JIL-01 with the value 3.33 compared to 3.30 on the field (Table 3). The initially classified NG-ZA-02, ZG-ZC-01, KN-MA-01, and KN-GU-02 as resistance accessions on the field, were susceptible to the pathogen under screen house evaluation with severity score of 6.33, 4.33, 5.67 and 5.00, respectively. However, all the susceptible accessions used as check have a value of greater than five (5), with NGB501 having a severity score of 7.33 and KD-CK-01 with 5.33 (Table 3).

Table 2. Blast severity on 35 Pearl Millet accessions under field condition.

Accessions	*Disease Scores	PDI	Significant Level	Host Reaction
NGB501	4.60	57.50	0.063	MS
NGB514	5.20	65.00	0.136	MS
NGB523	7.33	82.50	0.064	HS
NGB528	7.44	83.75	0.150	HS
NGB571	2.20	27.50	0.088	MR
NGB575	6.50	81.25	0.121	S
NGB578	6.10	76.25	0.083	S
NGB589	3.10	38.75	0.059	MR
NGB594	4.44	50.00	0.063	MS
NGB606	3.50	43.75	0.077	MR
NG-ZA-01	4.40	55.00	0.063	MS
NG-ZA-02	3.00	37.50	0.059	MR
NG-ZA-05	5.90	73.75	0.083	S
NG-ZA-08	1.90	23.75	0.060	R
NG-ZB-01	1.50	18.75	0.060	R
NG-ZB-03	6.20	77.50	0.083	S
NG-ZC-01	0.90	11.25	0.195	HR
NG-ZC-02	4.50	56.25	0.063	MS
NG-ZC-03	6.40	80.00	0.121	MS
KD-KG-01	4.70	58.75	0.136	MS
KD-CK-01	6.90	86.25	0.083	S
KD-JB-01	7.70	96.25	0.064	HS
KD-JM-01	2.30	28.75	0.088	MR
NS-JIL-01	3.30	41.25	0.055	MR
NS-YEL-02	0.80	76.25	0.195	HR
NS-YEL-06	4.50	56.25	0.063	MS
NS-YEL-07	2.10	26.25	0.088	MR
NS-GAN-04	4.60	57.50	0.052	MS
NS-GAN-05	4.33	48.75	0.063	MS
NS-GIN-03	0.78	8.75	0.195	HR
KN-MA-01	2.00	25.00	0.088	MR
KN-GU-02	2.50	31.25	0.088	MR
JIG-DU-01	5.25	52.50	0.136	MS
JIG-BIR-01	4.78	53.75	0.136	MS
ZF-ZM-01	5.60	77.50	0.054	MS
Mean	4.21	53.28		
% CV	47.56	44.25		

Values are mean of 30 replicate samples. Disease score of < 1.0 Highly Resistant (HR), 1.0-1.9 Resistant (R), 2.0-3.9 Moderately Resistant (MR), 4.0-5.0 Moderately Susceptible (MS), 5.1-7.0 Susceptible (S) and 7.1-9.0 Highly Susceptible (HS). PDI – Percentage Disease Incidence.

Screen house evaluation for blast infection

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accession NS-YEL-07 (0.67) and the least resistance being NS-JIL-01 with the value 3.33 compared to 3.30 on the field (Table 3). The initially classified NG-ZA-02, ZG-ZC-01, KN-MA-01, and KN-GU-02 as resistance accessions on the field, were susceptible to the pathogen under screen house evaluation with severity score of 6.33, 4.33, 5.67 and 5.00, respectively. However, all the susceptible accessions used as check have a value of greater than five (5), with NGB501 having a severity score of 7.33 and KD-CK-01 with 5.33 (Table 3).

Table 3. Blast severity of selected Pearl Millet accession under artificial inoculation.

Accession	Blast Severity		Host Reaction	
	Field	Screen House	Field	Screen House
*NGB501	4.60	7.33	MS	HS
NGB571	2.20	3.00	MR	MR
NGB589	3.10	2.33	MR	MR
NGB606	3.50	1.00	MR	R
NG-ZA-02	3.00	6.33	MR	S
NG-ZA-08	1.90	3.67	R	MR
NG-ZB-01	1.50	0.33	R	HR
NG-ZC-01	0.90	4.33	HR	MS
KD-JM-01	2.30	1.00	MR	R
*KD-CK-01	6.90	5.33	S	S
NS-YEL-02	0.80	0.00	HR	HR
NS-YEL-07	2.10	0.67	MR	HR
NS-JIL-01	3.30	3.33	MR	MR
NS-GIN-03	0.78	3.18	HR	MR
KN-MA-01	2.00	5.67	MR	S
KN-GU-02	2.50	5.00	MR	S

* = Susceptible check. Values are mean of 20 replicate samples. Disease score of < 1.0 Highly Resistant (HR), 1.0-1.9 Resistant (R), 2.0-3.9 Moderately Resistant (MR), 4.0-5.0 Moderately Susceptible (MS), 5.1-7.0 Susceptible (S) and 7.1-9.0 Highly Susceptible (HS).

Molecular analysis of selected accessions of pearl millet

RAPD primers performance and genetic diversity pattern

Fifty nine (59) amplified fragment loci were generated with 10 DNA primers from the selected 16 accessions used (Figure 1, Table 4). Out of the 59 fragment amplified bands, 53 (89.83%) were polymorphic and 6 (10.17%) were monomorphic. Primers T17 had the highest polymorphic amplification fragment of 8 bands, and highest monomorphic amplification fragment of 2 bands was detected by primer H10 and primers T17. However, 100 percentage polymorphism was recorded from primer B08, primer H04, primer T10, primer T01, primer T06 and primer T05 with polymorphic information contentment (PIC) of 0.91, 0.48, 0.85, 0.11, 0.87 and 0.87, respectively. The PIC was in the range of 0.11 – 0.91 with an average value of 0.72 (Table 4). However, there is no specific marker for resistant and susceptible accession to the disease.

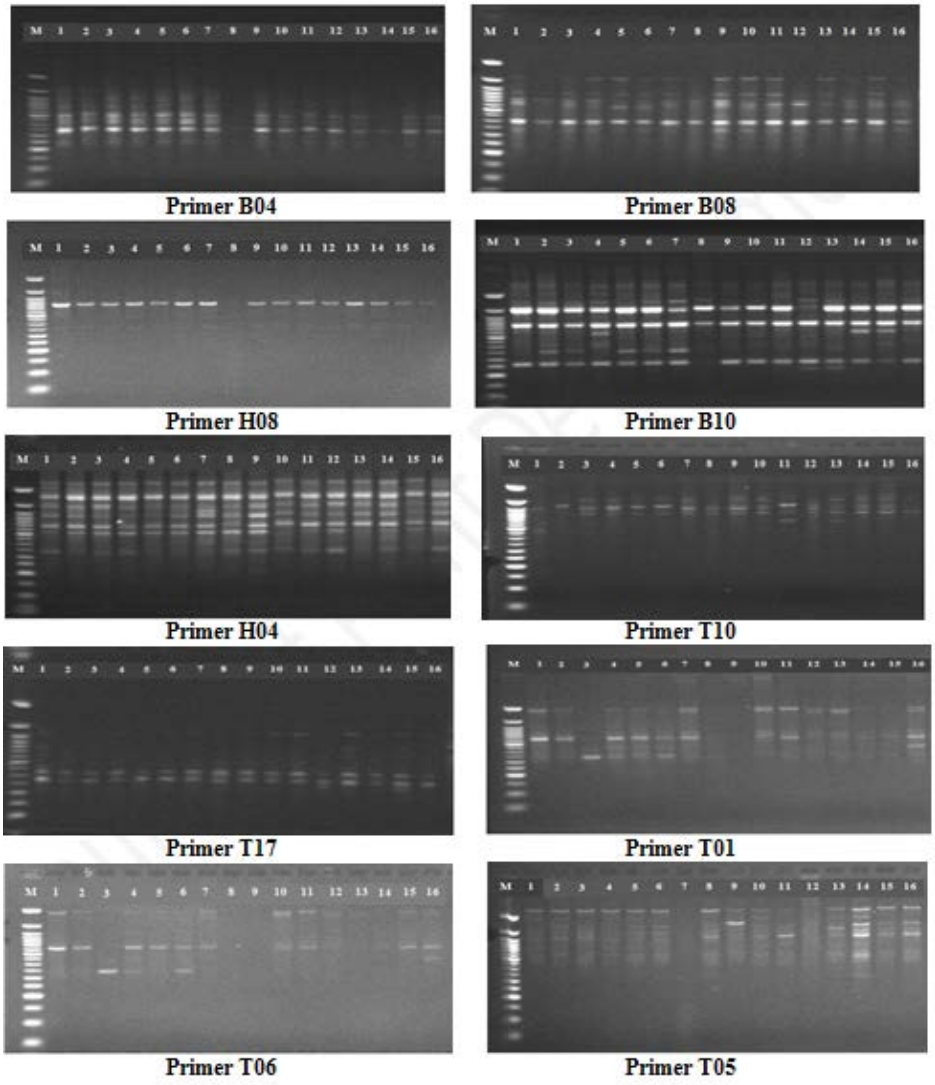

Figure 1. RAPD Amplification Patterns Using 10 Different DNA Primers.
M = ladder and number 1 to 16 on the first row represent the pearl millet accessions
1 NG-ZA-02, 2 NGB589, 3 NG-ZB-01, 4 KN-MA-01, 5 KN-GU-02, 6 NS-YEL-07, 7 NG-ZC-01, 8 NGB606, 9 KD-JM-01, 10 NG-ZA-08, 11 NS-YEL-02, 12 KD-CK-01, 13 NGB501, 14 NS-GIN-03, 15 NGB571, 16 NS-JIL-01.

Table 4. Sequencing and Polymorphic Information of ten RAPD DNA Markers for Pearl Millet.

Marker	DNA Sequence	Number of Monomorphic Band	Number of Polymorphic Band	Percentage Polymorphic Bands	PIC
primer B04	5^1 – GGA CTG GAG T - 3^1	1.00	6.00	85.71	0.86
primer B08	5^1 – GTG CAC ACG G - 3^1	0.00	6.00	100.00	0.91
Primer H08	5^1 – GAA ACA CCC C- 3^1	1.00	6.00	85.71	0.85
primer H10	5^1 – CCT ACG TCA G - 3^1	2.00	3.00	60.00	0.54
primer H04	5^1 – GGA AGT CGC C - 3^1	0.00	4 .00	100.00	0.48
primer T10	5^1 – CCT TCG GAA G - 3^1	0.00	7.00	100.00	0.85
primer T17	5^1 – CCA ACG TCG T - 3^1	2.00	8.00	80.00	0.90
primer T01	5^1 – GGG CCA CTC A - 3^1	0.00	1.00	100.00	0.11
primer T06	5^1 – CAA GGG CAG A - 3^1	0.00	6.00	100.00	0.87
primer T05	5^1 – GGG TTT GGC A - 3^1	0.00	6.00	100.00	0.87
Mean		**0.60**	**5.30**	**91.14**	**0.72**

A total of 88 alleles were generated by the 10 primer used, with the number of allele per primers ranges from 2.00 to 13.00 and overall average value of 8.80. Primer T17 had highest number of alleles (13.00) and primers T01 had the lowest with the value of 2.00 (Table 5). The major allelic frequency was highest in primer T01 (0.94) and the least was obtained from primer B08 with the values 0.13. Gene diversity ranged from 0.12 - 0.91 with a mean value of 0.74. The highest gene diversity (0.91) was generated by both primerB08 and primer T17, followed by primer T06 and primer T05 both with gene diversity of 0.88 and the least was recorded in primer T01 with the value of 0.12. With the exception of primer H10, primer H04 and primer T01, the gene diversity generated from each accession was higher than the overall average value of 0.74 (Table 5).

Dissimilarity and cluster analysis of pearl millet accession

The genetic similarity among the sixteen accessions of pearl millet varied between 0.18 and 1.44. The lowest similarity of 0.18 was for KN-MA-01 and KN-GU-02, and highest similarity of 1.44 was due to NGB606 and NG-ZA-08. The genetic similarity between KD-CK-01 and NGB501 was 0.29, but wide apart from all other accessions (Table 6). The UPGMA dendrogram generated from the 16 accessions revealed two major cluster groups (Figure 2). The first cluster (Group A) consisted of two susceptible accessions (KD-CK-01 and NGB501) with similarity coefficient 1.14. The second cluster group B, consist of all the 14 selected resistant accessions with only NS-YEL-02 sub-clustered in BI as a unique accession. Sub-clustered group BII, had the highest number of 13 accessions equivalent to 81.25% of the total selected accession and further sub-divided into two subgroups (C1 and C2) with similarity coefficient of 0.02. Sub-cluster C1 had accession two accessions (NG-ZA-08 and NS-JIL-01) with similarity coefficient value of 1.10. Sub-cluster C2 was further grouped into two sub-sub groups (C2a and C2b) with 6 accessions (KN-GU-02, NS-YEL-07, NG-ZC-01, KN-MA-01, NG-ZA-02 and NGB589) and 5 accessions (NG-ZB-01, NGB606, KD-JM-01, NS-GIN-03 and NGB571) respectively at

similarity distance coefficient of 0.01. However, the blast resistant accession NS-YEL-07 (Presented earlier in Table 3) was cluster in C2aI alongside with the susceptible accessions under the screen house conditions, while NG-ZA-02 and NGB589 were clustered together in a distinct group (C2aII). Cluster C2bI were all resistant accessions, with the high resistant accession NG-ZB-01 having the highest similarity distance coefficient (1.11) from its closest relative (Figure 2). This is an indication that RAPD molecular marker is an excellent tool in identifying and characterizing blast resistant and susceptible genotypes.

Table 5. Numbers of alleles, frequency and gene diversity of ten RAPD markers for Pearl Millet.

Marker	Sample size	Number of observable	Availability	Allele number	Major Allelic frequency	Gene diversity
primer B04	16.00	16.00	1.00	11.00	0.25	0.88
primer B08	16.00	16.00	1.00	13.00	0.13	0.91
primer H08	16.00	16.00	1.00	10.00	0.25	0.87
primer H10	16.00	16.00	1.00	5.00	0.63	0.57
primer H04	16.00	16.00	1.00	4.00	0.63	0.54
primer T10	16.00	16.00	1.00	10.00	0.25	0.86
primer T17	16.00	16.00	1.00	13.00	0.19	0.91
primer T01	16.00	16.00	1.00	2.00	0.94	0.12
primer T06	16.00	16.00	1.00	10.00	0.19	0.88
primer T05	16.00	16.00	1.00	10.00	0.19	0.88
Mean	16.00	16.00	1.00	8.80	0.36	0.74

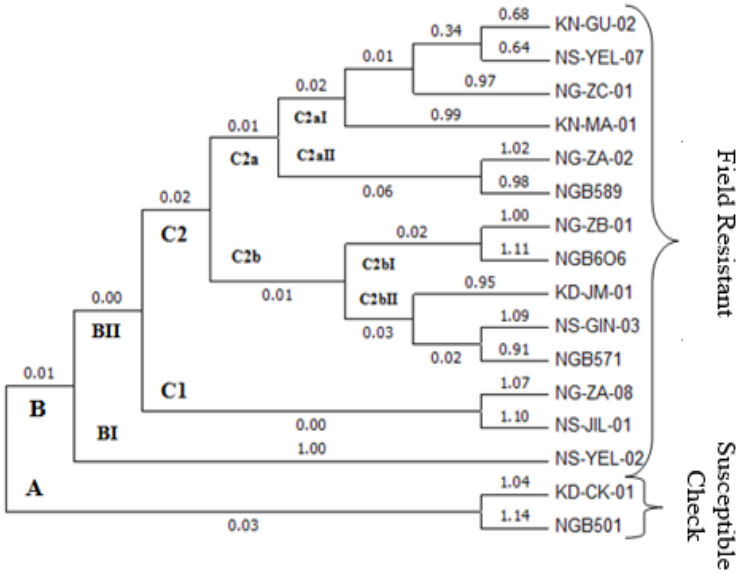

Figure 2. Dendrogram of genetic diversity of selected accessions of Pearl Millet.

Table 6. Dissimilarity matrix of sixteen (16) selected Pearl Millet accessions

X	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	0.00															
2	0.23	0.00														
3	0.43	0.39	0.00													
4	0.29	0.26	0.21	0.00												
5	0.47	0.29	0.36	0.18	0.00											
6	0.52	0.32	0.26	0.26	0.09	0.00										
7	0.52	0.39	0.47	0.26	0.29	0.32	0.00									
8	0.94	0.57	0.47	0.84	0.94	0.68	0.84	0.00								
9	0.47	0.52	0.23	0.36	0.47	0.36	0.36	0.76	0.00							
10	0.36	0.57	0.57	0.39	0.52	0.57	0.32	1.44	0.36	0.00						
11	0.29	0.47	0.47	0.39	0.36	0.39	0.47	0.68	0.43	0.26	0.00					
12	0.52	0.68	0.39	0.57	0.94	0.84	0.47	0.68	0.52	0.47	0.26	0.00				
13	0.47	0.62	0.62	0.62	0.68	0.62	0.43	0.62	0.57	0.36	0.29	0.29	0.00			
14	0.57	0.52	0.62	0.62	1.07	0.76	1.23	0.52	0.39	0.52	0.43	0.76	0.68	0.00		
15	0.43	0.39	0.39	0.32	0.43	0.39	0.57	0.68	0.23	0.47	0.32	0.39	0.62	0.23	0.00	
16	0.26	0.43	0.43	0.43	0.47	0.62	0.43	1.23	0.47	0.29	0.29	0.43	0.57	0.57	0.43	0.00

1 NG-ZA-02, 2 NGB589, 3 NG-ZB-01, 4 KN-MA-01, 5 KN-GU-02, 6 NS-YEL-07, 7 NG-ZC-01, 8 NGB606, 9 KD-JM-01, 10 NG-ZA-08, 11 NS-YEL-02, 12 KD-CK-01, 13 NGB501, 14 NS-GIN-03, 15 NGB571, 16 NS-JIL-01.

Discussion

Effective resistance breeding programmes require close monitoring of virulence changes in the pathogen and identification of new resistance sources to the virulent strains [THAKUR & al. 2009a]. Identification of fourteen (14) potential resistant genotypes under natural field condition and the confirmation of ten (10) of the genotypes under artificial screen house condition indicated that sources of resistance genotype(s) to blast pathogen in pearl millet could be obtained from the natural eco-type Germplasm and landraces. The variability in the level of resistance of the plants may be attributed to either their anatomical and/or genetical characters, which prevented the pathogen from penetrating and causing infection in the leaves. This result is in agreement with the work of SHANMUGAPACKIAM & RAGUCHANDER (2018), they identified 32 germplasm accessions from the mini-core collection having resistance to at least one of the five pathotypes of *Magnaporthe grisea* in India. According to WILSON & HANNA (1992), resistance expression in pearl millet to *Pyricularia* leaf spot has a tendency to be dominant or partially dominant. The susceptibility of some of the field potential resistance accessions to the pathogen under artificial screening, indicate their opportunistic resistance and could be favoured by the prevailing environmental condition at that period. This conformed to the report of THAKUR & al. (2009b), that screening under natural infection condition may give room for escapes, with variation in the level occurrence of the disease severity from season to season, thereby complicating the identification of the true resistance genotypes. It has been documented that with proper management of the crop, most diseases can escape or withstand with insignificant impact on yield [KHAIRWAL & al. 2007]. Also, in accordance

with these statements THAKUR & al. (2008, 2009) reported that under natural ecosystems, factors such as environmental variables, inoculum load and agronomic practices greatly influenced host-pathogen interaction thus, optimal for disease development and variation in the resistance of the cultivar.

The average polymorphic bands of 5.30 per primer obtained in this study was similar to 5.35 polymorphic bands per primer reported among 36 cultivars of six major cereal crops by CHAUHAN & al. (2015). These values were lower than the 6.30 of polymorphic band per primer reported by MOHAMMED & HAMZA (2018) using 40 accessions of pearl millet from Sudan and higher than 4.40 per primer reported by NWEKE (2014), who evaluated genetic diversity in 10 genotypes of pearl millet for drought tolerance using RAPD technique. The slight difference recorded among the results could be attributed to the variation in the number of sample used in the various studies. The mean polymorphic information content (PIC) of 0.72 obtained for all the RAPD markers used, indicate the effectiveness of the markers. AHMAD & al. (2015), reported that marker is effective if the PIC value is higher than 0.5. The ranged of polymorphic information content (0.48 - 0.91) recorded in the present study fall within the range of 0.48 - 0.76 reported by BENEDICT & al. (2016), 0.05 to 0.96 by KAPILA & al. (2008) using SSR markers.

According to PFEIFFER & al. (2011), higher number of alleles and high polymorphism are very important for correct estimation of genetic diversity of a germplasm and effectiveness of markers development, construction of segregating populations and provides enriched gene resources for gene mining in the grass family [WANG & al. 2012]. The high number of allelic mean (8.80) and wide range of gene diversity (0.12 - 0.91) with average value of 0.71 generated by the primers for the selected accessions indicate the level of diversity among the accession and reliability of the primers. The obtained mean value, agreed with the value of 0.74 across west and central pearl millet inbreds reported by STICH & al. (2010), 0.77 for Sudanese cultivated accessions [BASHIR & al. 2014], using SSR markers but, higher 0.49 among landraces from Niger [MARIAC & al. 2006] and 0.41 obtained for the 114 cultivated accessions from Benin, 0.49 in the cultivated [BENEDICT & al. 2016] using SSR markers. Thus, Nigeria and Sudan are probably the centres of origin of pearl millet.

The high polymorphic information content alongside with high gene diversity generated by the primers from the accession could be attributed to the free cross pollination of the species which result in high reshuffling of genes and its heterozygosity. This assertion is in line with the Hardy and Weinberg equilibrium principle which assumed that randomized mating population system result in heterozygosity of gene. In conformity with this statement, MOHAMMED & HAMZA (2018), opined that for pearl millet landraces, heterogeneity and heterozygosity at many loci within the accession is expected to be high due to the cross-pollinated nature of the crop, and their protogynous flowering behavior [ANIMASAUN & al. 2015]. The wide range of dissimilarity distance obtained in this study could be attributed to the difference in the geographic origin and pedigrees. However, pedigree information or geographic origins of cultivars may not accurately reflect the genetic relatedness among genotypes, whereas DNA markers could do when sufficient markers used are distributed across all chromosomes [ZHANG & al. 2005].

Information on genetic diversity and relationship among breeding materials in any germplasm collection had been reported to be important in identification of cultivars and selection of parents for hybridization, as well as predicting favorable heterotic combinations [KITAVI & al. 2014]. The clusters of accessions into resistant and susceptible

group indicate that random amplified polymorphic DNA (RAPD) markers could serve as selectable marker in pearl millet. This clustering of the accessions could be attributed to the presence of monogenetic disease resistance gene in pearl millet for blast as earlier shown by preliminary investigation of GUPTA & al. (2012), that pearl millet had single dominant gene for resistance to blast disease. In contrast to the result of this study, KITAVI & al. (2014) reported that distribution of species in a cluster is usually based on the spatial structure of the genetic variation across their geographical region. The variation among these reports could be due the differences in the sample size of the accessions used and number of primers. In line with these statements, YOUNG & KELLY (1996) had earlier reported that molecular markers such as RAPD, has the potential to serves as selectable marker where monogenic disease resistance genes have been identified. Identification of RAPD markers tightly linked to resistance genes has been reported to be important in selection of the resistance gene indirectly, since the expression of the molecular marker is not masked by epistatic interactions that take place between resistance genes [KELLY, 1995]. Similar to the observation of MAHATMA & al. (2011), the RAPD analysis does not link any specific marker for disease resistant and susceptible landraces. The non-specificity of any of the marker indicates their non-reliability for selection of resistance gene(s). The clusters of accessions from different state and sources in the same group by RAPD markers confirmed that there was no association between pattern of clusters and geographical distribution of accessions in this population. BENEDICT & al. (2016) attributed this clustering of accessions to wide germplasm exchange that occurs between farmers from different environment and even among regions due to the dependent of some regions on the main producers of the crop for their seeds. The result obtained further buttressed the fact that RAPD molecular marker is an excellent molecular tool in evaluating the genetic diversity among pearl millet accessions.

Conclusions

Considerable high variability exists among landraces of pearl millet in Nigeria from which resistance genotype(s) to blast pathogen could be obtained. Variation in the resistance and susceptibility of the accessions both on the field and nursery screening, demonstrated the inherent resistant potentials of some accessions as sources of resistance to blast disease. Based on the resistance capability, NS-YEL-02 could be selected as elite parent genotype in blast disease resistance breeding programs of pearl millet. Clustering of the selected landraces base on resistance and susceptibility by RAPD techniques, indicate its possibility for indirect selection of blast resistant genotypes for the crop. However, further genotyping is needed to identify the blast resistance gene(s) in the crop.

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EUPHORBIO VALDEVILLOSOCARPAE-INULETUM SALICINAE ASS. NOVA PÎNZARU, CANTEMIR & JARDAN (*TRIFOLION MEDII* T. MÜLLER 1962) IN THE REPUBLIC OF MOLDOVA

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Abstract: The vegetation of the "Peacock" glades in the "Codru" Scientific Reserve, Republic of Moldova, based on 15 relevés has been grouped in a new association *Euphorbio valdevillosocarpae-Inuletum salicinae* Pînzaru, Cantemir et Jardan, ass. nova, h.l., alliance *Trifolion medii* T. Müller 1962, ord. *Origanetalia vulgaris* T. Müller 1962, cl. TRIFOLIO-GERANIETEA SANGUINEI T. Müller 1962. The association consists of mesophilic phytocenoses, formed on slightly acidic, typical gray soils, at an altitude of 330-336 m. Hemicryptophytes predominate in the phytocoenoses of this association (74.1%), among the more numerous floristic elements, there are the Eurasian ones (53.7%), followed by the European ones (16.6%) and the Central European ones (6.4%).

Keywords: characteristic species, ecology, *Euphorbio valdevillosocarpae-Inuletum salicinae* ass. nova, range, Republic of Moldova.

Introduction

The vegetation of glades in the Republic of Moldova, for the most part, was studied from a phytosociological point of view, without identifying the plant associations, except for the glades in the arid cliff forests, which consist of phytocoenoses grouped in the association *Inulo ensifoliae-Anthericetum ramosi* Pînzaru et Coldea 2006 em. Pînzaru 2016, 2017. This article describes a new association – *Euphorbio valdevillosocarpae-Inuletum salicinae*, from the "Peacock" glades of the "Codru" Scientific Reserve. The "Peacock" glades are located on high hills, in the plots 43 and 52, surrounded by sessile oak forests (*Quercus petraea*) near Stejăreni village, Străşeni district.

Inula salicina L. (Figure 1) is a hemicryptophyte, Eurasian species, (xeromesophilic-) mesophilic (-mesohygrophilic), occurs in riverside meadows, glades and forest clearings, from hilly to mountainous areas, being part of the floristic composition of various associations. The association *Agropyro elongatae-Inuletum salicinae* Şerbănescu 1965 (Al. *Plantagini salsae-Artemision santonicae* Sheleag-Sosonko et Solomakha in Lysenko, Mucina et Iakushenko 2011) [DUBYNA & al. 2019] is found in the meadow vegetation of Ukraine, and the association *Violo elatioris-Inuletum salicinae* Didier et Royer 1989 (Al. *Molinion caeruleae* Koch 1926) [BENSETTITI & al. 2005] occurs in the hilly meadows of France. In the vegetation of Romania, there are no associations of *Inula salicina*, but it occurs as an accompanying species in other associations [COLDEA & al. 2012; CHIFU & al. 2014].

The characteristic species *Euphorbia valdevillosocarpa* Arvat et Nyár. [=*E. volhynica* auct. mold. non Besser ex Racib.] (Figure 2) is a Central European geoelement (endemic), occurring in Romania, the Republic of Moldova and Ukraine (western part). It is

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a mesophile and grows in glades, forest edges and sparse forests on hilly terrain [GELTMAN, 1996; SÂRBU & al. 2013]. In the Republic of Moldova, it is rarely found, it has been observed that it is somewhat more common (abundance + coverage from + to 2 and constancy V) in the phytocenoses of *Inula salicina* in the "Peacock" glades.

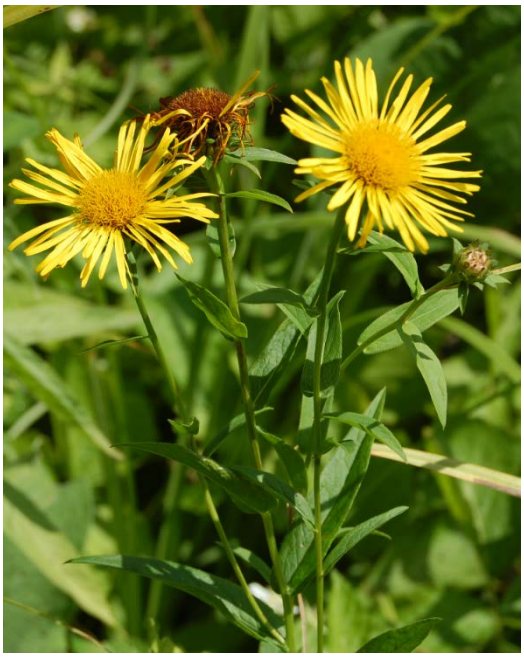

Figure 1. *Inula salicina* L.

Figure 2. *Euphorbia valdevillosocarpa* Arvat et Nyár.

Materials and methods

The phytosociological research was conducted in June-September, 2020, according to the Braun-Blanquet approach [BRAUN-BLANQUET, 1964]. The area of the relevés was 100 m^2 [CRISTEA & al. 2004]. Species nomenclature followed PÎNZARU & SÎRBU, 2016. The average annual temperature and precipitation were indicated according to the Atlas of Climate Resources of the Republic of Moldova [NEDEALCOV & al. 2013]. The soils – according to the monograph "The Soils of Moldova" [URSU, 2011].

Results and discussions

The plant communities of *Inula salicina* L. with *Euporbia valdevillosocarpa* Arvat et Nyár. and other species, in the "Peacock" glades, occur on slightly humic and slightly acidic typical gray soil, at an altitude of 330-336 m. The height of the hills and the slightly acidic soil create favourable conditions for the development of species characteristic of the class MOLINIO-ARRHENATHERETEA Tx. 1937, such as: *Briza media*, *Hypochaeris maculata*, *Ornithogalum pyrenaicum*, *Serratula coronata*, *Serratula tinctoria*, *Silene atropurpurea* etc.

These phytocoenoses have a compact coverage (100%), and a yellowish color predominates in the landscape during the flowering period of the dominant species.

Ass. *Euphorbio valdevillosocarpae-Inuletum salicinae*

Pînzaru, Cantemir et Jardan, ass. nova, hoc loco

Relevé type hoc loco: Table 1, rel. 6, N 47°05´536´´, E 028°27´242´´ (Figure 3).

Synoptic table hoc loco: Table 1, 15 relevés

The total area of the phytocoenoses of the association described in this article comprises about 2.6 ha.

Locations: Altitude: 330-336 m. Relief: Central Moldavian Plateau, on top of flat or slightly sloping hills (5°), with southern exposure. Soil: typical gray, slightly humic, slightly acidic, formed on loamy-clayey rocks. Climate: temperate-continental, the average annual temperature is 10.0-10.5°C, and the average annual precipitation varies between 650 and 700 mm.

Figure 3. As. *Euphorbio valdevillosocarpae-Inuletum salicinae* ass. nova (type) – 21 July 2020, Stejăreni village, Strășeni district.

Characteristic species: *Inula salicina*, *Euphorbia valdevillosocarpa*.

Constant species: *Centaurea jacea*, *Galium verum*, *Peucedanum cervaria*, *Tanacetum corymbosum*, *Serratula tinctoria*, *Achillea pannonica*, *Iris graminea*, *Filipendula vulgaris*, *Stachys officinalis*, *Briza media*.

Rare species: *Ornithogalum pyrenaicum* (= *O. flavescens* Lam.) [Endangered (EN)], included in the Red Book of Moldova (2015), *Serratula coronata* [Endangered (EN), included in the Red Book of Moldova (2015), *Silene atropurpurea* (= *Viscaria atropurpurea* Griseb.) [Critically Endangered (CR)], included in the Red Book of Moldova (2015), *Asparagus tenuifolius* (Least Consern (LC)] (Legea…1998), *Briza media* [Nearly Threatened (NT)] (Legea…1998), *Doronicum hungaricum* [Vulnerable (VU)], (Legea…1998), *Iris variegata* [Vulnerable (VU)] (Legea…1998), *Luzula campestris* [Nearly Threatened (NT)] (Legea…1998), *Orchis mascula* [Critically Endangered (CR)] (Legea…1998), *Hypochaeris maculata* L. [Vulnerable (VU)] (Legea…1998).

Structure: The overall vegetation cover is 100% (Figure 3). Although the plants in these phytocenoses are of different heights, from creeping to erect plants – about 150 cm tall, only the dominant species *Inula salicina* and *Euphorbia valdevillosocarpa* form a well-defined layer, reaching a height of 70-90 cm, the other species have an insignificant

abundance. The species of small plants (up to ±15 cm tall): *Viola odorata*, *Lysimachia nummularia*, *Melampyrum cristatum*, *Luzula campestris*, *Glechoma hirsuta*, *Fragaria viridis*, *Primula veris*, *Prunella vulgaris* etc., in some places, they have a cover between 5-10%, and the tall species (120-150 cm) have sporadic distribution: *Peucedanum cervaria*, *P. alsaticum*, *Thalictrum lucidum*, *Serratula coronata*, *Cirsium pannonicum*.

Floristic composition. In the 15 studied relevés, 108 species of vascular plants have been identified, and 47 of them are characteristic of coenotaxa of the class TRIFOLIO-GERANIETEA SANGUINEI T. Müller 1962, 21 species – cl. MOLINIO-ARRHENATHERETEA Tx. 1937, 11 species – cl. QUERCO-FAGETEA Br.-Bl. et Vlieger in Vlieger 1937, 3 species – cl. CRATAEGO-PRUNETEA Tx. 1962, and 26 species – Variae syntaxa.

The spectrum of life forms includes: hemicryptophytes (H) = 80 species (74.1%), geophytes (G) = 10 species (≈9.3%), chamaephytes (Ch) = 4 species (3.7%), nanophanerophytes (Phn) = 4 species (3.7%), therophytes (Th) = 8 species (7.4%), hemitherophytes (TH) = 2 species (1.8%).

Figure 4. Locations of the ass. *Euphorbio valdevillosocarpae-Inuletum salicinae* in the Republic of Moldova

In the spectrum of geoelements, the Eurasian ones predominate (Eua) = 58 species (53.7%), followed by the European ones (Eur) = 18 species (16.6%) and Central European (Euc) = 7 species (6.4%), other geoelements are represented by 1 to 4 species.

According to the soil humidity indices, in the phytocoenoses of the given association, there are 56 mesophilic (ms) species (51.7%) and 52 xeromesophilic (xm) species (48.3%), for these reasons, we have included this association in the alliance *Trifolion medii* T. Müller 1962, order *Origanetalia vulgaris* T. Muller 1962.

Range (Figure 4). The phytocoenoses of the association *Euphorbio valdevillocarpae-Inuletum salicinae* occur in the glades of sessile oak forests (plots no. 43 and 52), near Stejăreni village, Strășeni district.

Territorial protection. The phytocoenoses of the above-mentioned association are protected on the territory of the "Codru" Scientific Reserve.

Conservation value. The plant communities of the highlighted association are of high conservation value; they are rare and include 10 rare, protected species, among them, there are 3 species that are listed in the Red Book of the Republic of Moldova (2015).

Table 1. Ass. *Euphorbio valdevillosocarpae-Inuletum salicinae* ass. nov.

Life form	Geoelements	Edafic humidity	Relevé no.	1	2	3	4	5	*6	7	8	9	10	11	12	13	14	15	K
			Altitude (m)	330	331	331	335	335	335	335	335	335	335	335	335	336	336	336	
			Aspect	S	S	S	-	-	-	-	-	-	-	-	-	-	-	-	
			Slope (°)	5	5	5	-	-	-	-	-	-	-	-	-	-	-	-	
			General coverage (%)	100	100	100	100	100	100	100	90	100	100	100	100	100	100	100	
			Surface of relevé	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
			Number of species	30	24	39	36	26	36	25	43	29	41	29	24	32	31	34	
			Charact. species																
H	Eua	ms	Inula salicina	4	4	4	4	4	4	3	4	4	3	4	4	4	4	3	V
H	Euc	ms	Euphorbia valdevillosocarpa	1	1	1	1	1	2	2	1	1	2	1	1	1	1	1	V
			Trifolion medii																
H	Eua	ms	Centaurea jacea	+	1	1	+	1	+	+	1	1	-	1	+	1	-	+	V
H	Eur	ms	Achillea pannonica	1	+	+	1	r	+	r	1	+	1	+	-	-	+	-	IV
H	Eua	ms	Lathyrus pratensis	+	+	-	+	+	+	-	-	+	-	-	+	+	+	-	III
H	Eur	ms	Knautia arvensis	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	I
H	Eua	ms	Leucanthemum vulgare	-	-	-	-	-	-	-	r	-	r	-	-	-	-	r	I
H	Eua	ms	Trifolium medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	I
			Origanetalia vulgaris																
H	Eua	ms	Galium verum	+	+	+	+	+	+	r	+	+	+	+	+	r	+	2	V
H	Eua	ms	Filipendula vulgaris	r	+	+	+	-	r	r	+	-	1	r	-	+	+	r	IV
H	Eua	ms	Galatella sedifolia	+	+	-	-	-	1	1	-	-	-	1	1	1	1	+	III
H	Eur	xm	Trifolium alpestre	-	-	-	+	+	+	r	+	-	r	-	-	r	+	+	III
H	Euc	xm	Valeriana collina	r	-	r	-	-	-	-	-	-	-	r	r	r	-	r	II
H	Eur	ms	Lathyrus sylvestris	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	I
H	Eua	xm	Medicago falcata	-	-	-	r	-	r	-	-	-	r	-	-	-	-	-	I
H	Eua	xm	Origanum vulgare	-	-	-	-	r	-	-	-	-	r	-	-	-	-	-	I
H	Eua	ms	Primula veris	-	-	r	-	-	+	-	-	-	-	-	-	-	-	-	I
H	Euc-M	xm	Securigera varia	-	-	-	-	+	-	-	-	-	r	-	-	r	-	-	I

H	Eua	ms	Silene vulgaris	-	-	r	-	-	-	-	r	-	-	-	-	-	-	-	I
H	Circ	xm	Solidago virgaurea	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	I
Th	Eua	ms	Vicia hirsuta	-	-	-	-	-	-	r	-	-	-	-	r	-	-	-	I
			Geranion sanguinei																
H	Eur	xm	Peucedanum cervaria	r	1	+	+	+	+	-	+	+	r	r	r	-	r	1	V
H	Euc	xm	Galium rubioides	-	+	-	-	-	-	-	-	r	r	-	-	r	r	r	II
Th	Eur	xm	Melampyrum cristatum	+	-	-	r	r	r	r	+	-	-	-	-	-	r	-	II
G	P-P-B	xm	Iris variegata	-	-	-	-	-	-	-	-	-	r	r	-	r	-	-	I
H	Euc	xm	Peucedanum alsaticum	-	-	-	-	r	-	-	-	-	r	-	-	-	-	-	I
H	Euc-M	xm	Prunella laciniata	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	I
H	Med	xm	Silene coronaria	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	I
H	Eua	xm	Trifolium montanum	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	I
H	Eua	xm	Veronica spicata	-	-	-	r	-	-	-	-	-	-	-	-	-	r	-	I
			Antherico ramosi-Geranietalia sanguinei																
H	Eua	xm	Tanacetum corymbosum	r	r	r	r	r	r	r	+	r	r	-	r	+	-	+	V
G	Pont-M	xm	Iris graminea	r	r	r	r	r	r	r	-	r	r	-	-	r	r	-	IV
H	P-P	xm	Cirsium pannonicum	-	-	-	-	-	-	-	+	r	r	-	-	r	r	+	II
TH	Med	xm	Arabis sagittata	-	-	-	-	-	-	-	-	-	r	-	-	r	-	r	I
H	Eua	xm	Nepeta nuda	-	-	r	-	-	-	-	r	-	-	-	-	-	-	-	I
			Trifolio-Geranietea																
H	Eua	ms	Stachys officinalis	+	-	r	r	r	-	r	r	-	r	r	r	r	r	r	IV
H	Circ	xm	Clinopodium vulgare	r	-	r	r	-	+	-	+	-	+	r	-	-	r	-	III
H	Pont	xm	Dianthus membranaceus	-	-	-	r	r	r	-	r	r	-	r	-	-	r	r	III
H	Eua	xm	Veronica teucrium	r	+	r	r	r	r	r	r	r	-	-	-	-	-	-	III
H	Eua	xm	Campanula glomerata var. cervicarioides	-	-	-	-	-	-	-	-	r	r	-	-	r	r	-	II
H	Eua	ms	Hypericum perforatum	-	-	r	-	-	-	r	r	-	r	r	-	-	-	r	II
H	Eua	ms	Vicia cracca	-	-	r	-	+	-	-	r	r	r	-	-	-	-	+	II
H	Eua	xm	Campanula persicifolia	-	-	-	-	-	-	-	r	-	r	-	-	-	r	-	I

Th	Eur	xm	Dianthus armeria	-	-	-	-	-	-	-	-	-	-	-	r	-	-	-	I
H	Eua	ms	Fragaria vesca	+	1	+	+	-	-	-	-	+	+	-	-	-	-	-	I
Ch	Euc-M	xm	Teucrium chamaedrys	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	I
H	Eur	xm	Vincetoxicum hirundinaria	r	-	r	-	-	-	-	-	-	-	-	-	r	-	-	I
			Molinio-. Arrhenatheretea s.l.																
H	Eua	ms	Serratula tinctoria	+	+	+	r	-	+	1	-	+	r	+	+	+	+	1	V
H	Eua	ms	Briza media	-	r	-	r	-	+	-	+	r	r	r	r	r	r	r	IV
H	Eua	ms	Dactylis glomerata	+	+	+	-	r	-	1	-	-	-	-	+	+	-	-	III
G	Euc-M	ms	Ornithogalum pyrenaicum	+	+	r	+	-	-	-	-	-	-	+	-	+	+	-	III
H	Eur	ms	Salvia pratensis	r	r	r	r	r	-	-	r	r	-	-	-	-	-	-	III
H	Eua	ms	Hypochaeris maculata	r	-	r	-	-	-	-	-	-	-	-	-	r	r	-	II
H	Pont	ms	Serratula coronata	-	-	r	-	-	-	-	-	r	-	r	-	r	r	-	II
H	Balc	ms	Silene atropurpurea	-	-	-	-	-	r	-	r	-	-	-	r	-	-	r	II
H	Euc-Po	ms	Thalictrum lucidum	r	r	-	-	-	-	r	-	-	r	+	r	-	-	-	II
H	Eua	ms	Calamagrostis epigejos	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	I
H	Eua	ms	Leontodon hispidus	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	I
H	Eua	ms	Lotus corniculatus	-	-	-	-	-	-	-	r	-	r	-	-	-	-	-	I
H	Eur	ms	Luzula campestris	-	-	-	-	-	+	-	-	-	-	-	-	-	-	r	I
Ch	Eur	ms	Lysimachia nummularia	r	-	+	+	-	-	-	-	-	-	-	-	-	-	-	I
H	Eua	ms	Plantago media	-	-	-	-	r	-	-	r	r	-	-	-	-	-	-	I
H	Circ	ms	Prunella vulgaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	I
H	Circ	ms	Scutellaria galericulata	-	-	-	-	-	-	+	-	-	-	-	-	-	r	-	I
H	Eua	ms	Stellaria graminea	-	-	-	r	-	-	-	r	-	-	-	-	-	-	r	I
H	Eua	ms	Veronica longifolia	-	r	-	-	-	-	-	-	-	-	-	-	r	-	-	I
H	Eua	ms	Viola jordanii	-	-	-	-	-	r	-	-	-	r	-	-	-	-	-	I
H	Eua	ms	Viola pumila	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	I
			Crataego-Prunetea s.l.																
Phn	Eur	xm	Crataegus monogyna	r	-	-	r	-	r	-	r	r	r	r	+	-	-	-	III
Phn	Eur	xm	Rosa canina	-	-	r	-	-	r	-	-	r	-	-	-	r	-	-	II

Phn	Eua	xm	Prunus spinosa	-	-	-	-	-	-	-	+	-	r	-	-	-	-	-	I
			Querco-Fagetea s.l.																
G	Pont	xm	Carex brevicollis	-	-	+	-	+	+	-	r	r	r	r	-	-	-	-	III
G	Pont-M	xm	Asparagus tenuifolius	-	-	r	r	-	-	-	r	-	-	-	r	r	-	-	II
H	Eua	ms	Ranunculus auricomus	-	-	r	r	r	-	-	r	-	-	r	r	-	-	-	II
H	Med	xm	Viola odorata	r	-	r	+	-	r	-	r	-	-	-	-	-	-	-	II
G	P-P-B	ms	Doronicum hungaricum	-	r	-	-	-	-	-	-	r	-	-	-	-	-	-	I
H	Pont-M	xm	Glechoma hirsuta	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	I
H	Eua	ms	Brachypodium sylvaticum	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	I
H	Eua	ms	Hypericum hirsutum	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	I
H	Euc	xm	Lathyrus niger	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	I
G	Eur	ms	Orchis mascula	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	I
Phn	Eur	xm	Pyrus pyraster	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	I
			Variae syntaxa																
G	Eua	ms	Elymus repens	1	1	1	+	-	1	-	1	1	1	2	1	1	1	-	IV
H	Eua	xm	Festuca valesiaca	+	-	-	+	+	+	-	1	-	+	-	-	-	+	+	III
Ch	Eua	xm	Artemisia austriaca	-	+	r	r	-	-	-	r	-	-	-	-	-	-	-	II
H	Eua	xm	Euphorbia virgata	-	-	-	+	-	-	+	+	-	-	+	-	-	-	+	II
H	Eur	ms	Ajuga reptans	-	-	r	-	-	-	-	-	-	-	-	-	-	r	-	I
G	Eua	ms	Allium oleraceum	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	I
Th	Eur	xm	Alyssum alyssoides	-	-	-	-	-	r	-	-	-	-	-	r	-	-	-	I
H	Eua	xm	Artemisia pontica	-	-	-	-	-	-	-	-	-	-	r	r	-	-	-	I
H	Eua	xm	Bromus inermis	-	-	-	-	-	-	+	-	-	-	r	-	-	-	r	I
Th	Eua	xm	Buglossoides arvensis	-	-	-	-	-	-	r	-	-	-	-	-	r	-	-	I
H	Eua	ms	Carex polyphylla	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	I
G	Eua	xm	Carex praecox	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	I
Th	Adv	ms	Erigeron annuus	-	-	-	-	-	-	-	-	-	r	-	-	-	-	r	I
H	Euc	xm	Koeleria pyramidata	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-	I
H	Eua	ms	Linaria vulgaris	-	-	-	-	-	r	-	-	-	-	-	-	-	-	+	I

H	Eua	ms	Phleum phleoides	-	-	-	-	-	-	-	+	-	r	-	-	-	-	-	I
H	Eua	xm	Pilosella bauchinii	-	-	-	-	-	r	-	r	-	-	-	-	-	-	r	I
H	Eua	xm	Poa angustifolia	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	I
H	Eur	xm	Polygala comosa	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	I
H	Eua	xm	Potentilla argentea	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	I
H	Eua	xm	Poetntilla recta	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	I
H	Eua	ms	Tanacetum vulgare	-	-	-	-	-	-	-	-	-	r	+	-	-	-	-	I
Ch	Eua	xm	Thymus pannonicus var. marschallianus	-	-	-	-	-	r	-	r	-	-	-	-	-	-	-	I
TH	Euc-M	ms	Tragopogon dubius	-	-	-	-	-	-	r	-	-	-	-	-	-	r	-	I
Th	Eur	ms	Valerianella locusta	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	I
Th	Med	ms	Trifolium campestre	-	-	-	-	-	-	-	+	-	-	-	-	-	-	r	I

Place and date of the relevés: 1-3, glade no. 1, plot no. 52, Stejăreni village, Strășeni district, 10.VI.2020, 21.VII.2020, 11.IX.2020; 4-12 (*6 -typus), glade no. 2, plot no. 43, Stejăreni village, Strășeni district, 10.VI.2020, 21.VII.2020, 11.IX.2020; 13-15, glade no. 3, polt no. 43, Stejăereni village, Strășeni district, 10.VI.2020, 21.VII.2020, 11.IX.2020.

Conclusions

The association *Euphorbio valdevillosocarpae-Inuletum salicinae* Pînzaru, Cantemir et Jardan ass. nova includes plant communities of hemicryptophytes (74.1%), mesophiles and xeromesophiles, formed on high hills (330-336 m altitude), on slightly acidic, typical gray soils.

In the floristic composition, the Eurasian elements predominate (53,7 %), followed by the European (16.6%) and Central-European ones (6.4%). The differential species *Euphorbia valdevillosocarpa* Arvat et Nyár. is a Central European geoelement (endemic), therefore the association can also be considered Central European (Eastern).

The association *Euphorbio valdevillosocarpae-Inuletum salicinae* Pînzaru, Cantemir et Jardan ass. nova has been included in the alliance *Trifolion medii* T. Müller 1962, order *Origanetalia vulgaris* T. Müller 1962, class TRIFOLIO-GERANIETEA SANGUINEI T. Müller 1962.

It has been proposed to include the association *Euphorbio valdevillosocarpae-Inuletum salicinae* in the List of Rare Plant Associations of the Republic of Moldova, with high conservation value.

Notes on contributors

Pavel PÎNZARU is a professor, PhD, botanist with a special interest in phytocoenology, floristics and systematics. The has published over 195 scientific papers on the flora and vegetation of Bessarabia and Italy. He is co-author on the "Red Book of the Republic of Moldova" (2001, 2015), the "Flora of Bessarabia volume" I, II, III (2011, 2016, 2020), is author the "Pteridophytes in the Republic of Moldova" (2019) and the "Rare plants in rocky regions of the Republic of Moldova" (2020).

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PROTOPLAST AT THE TIME OF GENOME EDITING

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Abstract: Many uses of protoplasts, plant cells with the cell wall removed, have been explored. Many advantages of the system have been realized and proven in recent years in various physiological, biochemical, genetic, and molecular biological studies. Reliable methods to isolate viable protoplasts from a broad variety of plant species have been established. Regeneration of plants from protoplasts has become one of the options involved in crop gene manipulation and crop improvement. Here, we present how protoplast system may help crop gene editing and novel trait development, and discuss the potentials and challenges of this approach.

Keywords: CRISPR, crop improvement, gene editing, protoplast, regeneration, transient expression.

Introduction

Crop improvement through genetic manipulation has been in practice for decades. T-DNA-based gene overexpression, RNAi, or transposon insertional mutagenesis play a significant role in the manipulation of gene expression levels or changes of phenotypes. However, there is an increasing demand for simultaneous multi-gene manipulations for two main reasons: (1) The current wealth of different data types annotating the genome and how the many molecules in the parts interact with each other poses a demand to develop methods of integration that seamlessly connect genome-wide data; (2) Multi-traits development such as value added food, enhanced stress and pathogen-resistant crops, energy efficient architecture and increased yield also requires tools for multi-targeting and multi-manipulation. In the past a few years, multiplex genome editing strategies have been developed and become available for such a need. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are proteins that can produce double-strand DNA breaks that when repaired introduce site-specific mutations or insertions [JAGANATHAN & al. 2018]. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system uses RNAs to target nucleases to specific sites; when repaired, site-specific mutations or insertions are introduced [JAGANATHAN & al. 2018]. Multiple single guide RNAs (sgRNAs) with various target sequences can also direct Cas9 to multiple sites [CONG & al. 2013]. This feature of Cas9 allows simultaneous editing of multiple loci in the same individual.

Transgene-based delivery systems and non-transgene delivery systems are both applied to gene-editing. In the latter, additional advantages exist in protoplast system. Protoplasts have been successfully, and in some cases, routinely applied to complex signaling analysis in many plant species including *Arabidopsis*, tomato, tobacco, broad bean, maize, rice, wheat, barley, poplar, petunia, and moss [XING & WANG, 2015]. Various plant tissues can provide the cells for protoplast production. Protoplasts have been isolated from suspension cultures, callus cultures, embryos, shoots, and seedlings [XING & WANG, 2015]. The versatile cell-based assays have significantly facilitated an integrated

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understanding of some complex mechanisms such as plant signaling network [SHEEN, 2001; LI & al. 2015; XING & WANG, 2015; XING & al. 2017]. In addition to the above two main demands, protoplast system is a critical alternative in crop engineering in that they can be regenerated into plants [WOO & al. 2015; LIN & al. 2018]. Here, we will highlight the significant opportunities and challenges for plant protoplast system in crop genome editing.

Protoplast and large-scale screening

Genomic data of model systems and crop species have provided us with overwhelming amount of resources and discovery of the function of any genes in the genome (e.g. 25,000 in *Arabidopsis* and 41,000 in rice) is now within reach. While stable transformation takes considerable amount of time, initial screening can be achieved in a cost-effective manner in protoplast system [JUNG & al. 2008; XING & WANG, 2015]. The system is applicable for the analysis of gene expression effect, knock-out or knock-in gene editing effect and protein-protein interactions [EHLERT & al. 2006; LI & al. 2011, 2015; XING & WANG, 2015; SAKAMOTO & al. 2020]. Further developed robotic systems for protoplast isolation and transformation facilitated automated high throughput screening [XING & al. 2014; LOWDER & al. 2015; QUÉTIER, 2016; ČERMÁK & al. 2017].

As tissue culture and regeneration procedures to generate gene-edited events are time consuming, large-scale screening will facilitate rapid validation of genome-editing reagents and screening for resulting targeted mutagenesis [DLUGOSZ & al. 2016; NADAKUDUTI & al. 2019]. In the past a few years, protoplasts were successfully applied to gene editing analysis in *Arabidopsis* [LI & al. 2013, 2015], tobacco [LI & al. 2013], maize [LIANG & al. 2014], brassica [MUROVEC & al. 2018], rice [SHAN & al. 2014], wheat [WANG & al. 2014b; ZHANG & al. 2016; LUO & al. 2019], soybean [SUN & al. 2015], tomato [ČERMÁK & al. 2015], potato [ANDERSSON & al. 2017], strawberry [GOU & al. 2020], grapevine and apple [MALNOY & al. 2016]. Transient protoplast transfection is also an alternative strategy to test multiple mutagenesis parameters rapidly [LIN & al. 2018]. Hence, transient assays using protoplasts from various plant species hold great promise for increasing the speed at which genes can be studied, bridging the gap between the large data sets coming from high-throughput assays and the time consuming and laborious *in planta* investigations. Protoplast is also one of the main plant materials for Cas9 system delivery in various studies of crop species [MANGHWAR & al. 2019].

Selection of plant materials

Protoplast generation involves removal of tissue surface and enzyme treatment. The protocols for *Arabidopsis* mesophyll or maize mesophyll protoplast systems and *Arabidopsis* or tobacco BY-2 suspension cultured cells could serve as guidelines [SHEEN, 2001; YOO & al. 2007]. A simpler protoplast isolation method involving the use of two different adhesive tapes to sandwich *Arabidopsis* leaves was also developed [WU & al. 2009]. One should evaluate the isolation success before moving to any analysis. Intact viable protoplasts could be identified by a few common methods including (1) viable protoplasts exclude Evan's blue appearing clear or yellowish against a blue background; (2) viable protoplasts can accumulate neutral red and turn red; (3) fluorescent dyes are also used to stain viable protoplasts [XING & WANG, 2015].

Use of healthy leaves at the proper developmental stage is a very important factor in the production of viable protoplasts from *Arabidopsis* while stressed leaves (e.g. those

under drought, flooding, extreme temperature and constant mechanical perturbation) may seemingly give protoplasts but they only lead to low transfection efficiency when used in gene expression analysis [YOO & al. 2007]. In a sense, protoplast isolation remains a bottleneck to testing genome-editing reagents in many crop species. It is critical in the study of gene-editing effect to give considerable time to set up a reproducible protoplast system.

Transfection efficiency

The plant species, source materials, isolation methods and transfection methods all play a role in determining the transfection efficiencies. From one case to another, this can vary dramatically, e.g. around 50 to 70% in tomato leaf mesophyll system [XING & al. 2001, 2008] down to only 5% to 20% in *Arabidopsis* root protoplasts [BARGMANN & BIRNBAUM, 2009]. The best so far probably is the *Arabidopsis* mesophyll protoplast system, which may reach 90% transfection efficiency [SHEEN, 2001; YOO & al. 2007]. However, further improvement in transfection efficiency is always possible in any systems. Protoplasts from six species in *brassicaceas* also gave 43-83% transfection efficiency [WU & al. 2009]. Transfection efficiency was also tested and improved in multiple species from a single study, where protoplast transfection efficiency was shown to be 44-63% for rice, maize, wheat, millet, bamboo, and tomato [LIN & al. 2018]. In this study, the efficiency of CRISPR/Cas9-mediated mutagenesis (insertions, deletions) in the isolated protoplasts from different species varied dramatically ranging from 0.2% and 1.1% for *Zea mays* to 75.2% in *Brassica oleracea* [LIN & al. 2018]. Co-expressing GFP along with site-specific nuclease (SSN)-reagents in protoplasts may help the detection of the delivery and expression of genome-editing reagents and the co-expression approach facilitated direct comparison of the transformation efficiencies of CRISPR/Cas9 and TALEN reagents [NADAKUDUTI & al. 2019]. Although protoplast transfection efficiency is not correlated to the efficiency of CRISPR/Cas9-mediated mutagenesis, a healthy population of protoplasts with a high transfection efficiency is critical for CRISPR/Cas9-mediated mutagenesis. It should be noted that there is considerable variability in gRNA efficiency, and this does not seem to change with expression system or Cas9 delivery method. The feasibility of improving CRISPR/Cas9 editing efficiency by Fluorescence Activated Cell Sorting (FACS) of protoplasts was examined and protoplasts expressing GFP tagged CRISPR/Cas9, delivered through *A. tumefaciens* leaf infiltration, could be enriched by FACS [PETERSEN & al. 2019].

Regeneration from protoplast

Started with gene editing in protoplast, whole plants were generated with targeted modifications for various plant species [LI & al. 2013; SHAN & al. 2014; WANG & al. 2014b; SUN & al. 2015; WOO & al. 2015; CLASEN & al. 2016; MALNOY & al. 2016; KIM & al. 2017; LIANG & al. 2017; MANGHWAR & al. 2019]. CRISPR/Cas12a and base editing systems along with DNA-free CRISPR delivery methods were also implemented in protoplasts, targeted mutagenesis achieved, and plants regenerated with desired edited mutations [WOO & al. 2015; ANDERSSON & al. 2017; KIM & al. 2017]. For plant species that can be regenerated from protoplasts, the phenotypic changes are assessed at the whole plant level. With high delivery efficiencies and effective nucleases, a significant number of the plants regenerated from transformed protoplast populations harbor mutations at the target locus [CLASEN & al. 2016; LI & al. 2016]. A majority of them were shown not to have foreign DNA, which indicate that the nuclease was expressed only transiently,

and the construct was degraded before integration. This certainly is an additional advantage as the lack of foreign DNA is often desirable due to biotechnology regulation.

Difficulties do exist, together with concerns. Protoplast regeneration is difficult in most plant species [LIN & al. 2018], particularly monocots [BERNARD & al. 2019]. The regeneration process is time-consuming and is often preferred for quick efficiency test of gene editing systems or the mutagenesis effect [SOYARS & al. 2018]. Biolistic or *A. rhizogenes*-mediated transformation could be a common alternative. *A. rhizogenes* has been widely used to study rhizosphere, metabolic or hormone pathways [XING & al. 1996; GOMES & al. 2019]. Knocking out the CiPDS gene (phytoene desaturase) in chicory plants regenerated from both hairy roots and protoplasts was successfully shown [BERNARD & al. 2019]. We could expect that the list of protoplast-generated crops to expand because of the merit of ribonucleoprotein (RNP)-based genome editing technology. The significant potential is that while transient expression screening in protoplasts provides information for short listing of genes, further functional analysis with gene editing approaches can be followed by regeneration of plants so that gene editing effect will be analyzed at organismal level (Figure 1).

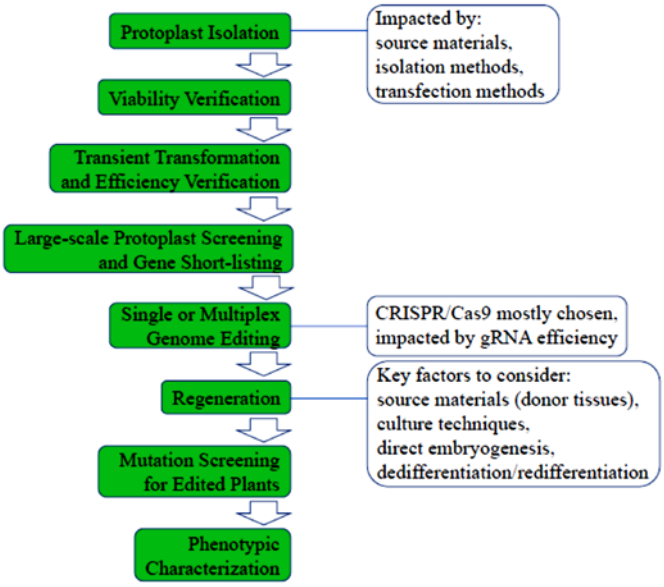

Figure 1. Schematic diagram of protoplast application for single or multiplex gene editing and plant regeneration. Some technique notes are indicated by boxes on right.

Conclusions

It is important that findings from large-scale omics analysis are confirmed by functional analysis. With the gene editing development such cell-based assays and functional screening will continue to facilitate the comprehensive understanding of the complexity of many processes in plants. Studies in the past several decades have indicated the usefulness of protoplasts and defined protoplast expression systems [XING & WANG,

2015]. Well established *Arabidopsis*, maize, tobacco, and tomato protoplast systems were applied to analysis of responses to oxidative, heat and osmotic stress signals, and pathogen elicitors [SHEEN, 1996; KOVTUN & al. 2000; TENA & al. 2001; XING & al. 2001, 2008; XING & WANG, 2015]. Protoplast system has also been applied to the analysis of developmental reprogramming [WANG & al. 2014a] and detailed metabolite investigation in specific cell types such as guard cells [JIN & al. 2013; RUBAKHIN & al. 2013]. As indicated in our previous work, a major application is protoplast transient transfection assay for the analysis of (1) gene expression in response to various signals and treatments; (2) promoter elements involved in regulating expression of genes; (3) roles played by signaling proteins such as protein kinases and transcription factors in regulating gene expression; (4) subcellular localization of proteins; (5) genetic interactions of genes; (6) protein-protein interactions; (7) gene interference effect; (8) proteomic and metabolomic profiles; and (9) functions of large number of genes derived from large-scale studies as an initial screening process [XING & WANG, 2015]. With such a broad application to mechanism analysis, we could be confident that the protoplast system will play an increasingly significant role in the coming years when high-throughput approaches and gene editing approaches meet.

Notes on contributor

Tim XING is an associate professor and a plant molecular biologist with a special interest in cell signaling and plant-microbe interactions. He teaches plant physiology, molecular plant development, and cell signaling.

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The 80th Anniversary of the Biologist Rodica RUGINĂ

Rodica Rugină was born in Clipiceşti, Vrancea county, on 20th of January, 1940. The education provided by her parents, the teachers Vasile and Paraschiva Surugiu, set the basis of her continuous process of formation and training as future botanist. Her educational path started in his native village, where she attended primary school, and continued in Focşani, where she attended lower and upper secondary school.

She successfully completed her high school education in 1958 by getting the Baccalaureate diploma and then got admitted by exam to the "Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology and Geography, Biology - Botany Department.

As a student, Rodica Surugiu proved the qualities necessary for a future researcher (seriousness, diligence, passion for the research work), especially by participating in the botany circle. She completed her university studies in 1963 with the BA degree.

Mrs. Rodica Rugină's professional career was closely linked to three representative institutions of Iaşi: the University of Medicine and Pharmacy, the "Al. I. Cuza" University and the Botanical Garden.

Thus, between 1963 and 1967, she worked as assistant at the Pharmaceutical Botany Department of the Faculty of Pharmacy of Iaşi, having obtained the job governmental decision. Probably it was there where she gained a special interest in medicinal plants, which she studied a lot in the following years. Later on she transferred to the Faculty of Biology and Geography of the "Alexandru Ioan Cuza" University, working as assistant at the Plant Morphology Department until 1968. During this period she also began doctoral studies (1971) with the thesis "Morphological and histo-anatomical research for regular weeds and those modified by herbicide treatment", under the supervision of Professor Constantin Burduja. The thesis was defended publicly in 1980, so the passionate botanist became PhD in Biology.

Due to the reorganization of the Faculty of Biology, Rodica Rugină becomes a researcher within the Botanical Garden of the University, where she coordinated the activity of the Taxonomic Department, but in all her years of activity (1968-2002), Rodica Rugină continued to collaborate with the members of the morphology and plant anatomy team of the Faculty of Biology.

Throughout her professional career, Rodica Rugină carried out an intense didactic activity, conducting practical laboratory work (Pharmaceutical Botany, Morphology and Plant Anatomy) and field practice, supervising theses, dissertations and promotion scientific papers in highschool teaching.

The scientific activity, carried out mainly in collaboration with the members of the Plant Morphology Department, resulted in the publication of over 90 scientific articles, in

various research fields: comparative anatomy (systematic), ecological anatomy and experimental anatomy, with special attention to protected and medicinal plant species. Probably her most valuable work, that took her many years of intense study, is *Anatomy of medicinal plants. Atlas*, published in 1998, in joint authorship with Professor Constantin Toma, at the prestigious publishing house of the Romanian Academy. For this work, in 2000, Rodica Rugină was awarded the "Emanoil Teodorescu" Prize by the Romanian Academy.

Rodica Rugină has published, alone or by collaboration, other books of botanical interest: *Can the mysteries of life be solved?* (V. Rugină and R. Rugină, 1993), *Protected plants in Romania* (R. Rugină and M. Mititiuc, 2003), *Volatile oils and aromatherapy* (R. Rugină and I. Boz, 2013), *The adventure of plants on the globe* (R. Rugină, 2017). In the same way she contributed to the publication of manuals of practical works of Morphology and anatomy of plants, as well as to the work *Illustrated flora of vascular plants in Eastern Romania* (Sârbu I., Ştefan N., Ivănescu L., Mânzu C., vol. 1, 2001).

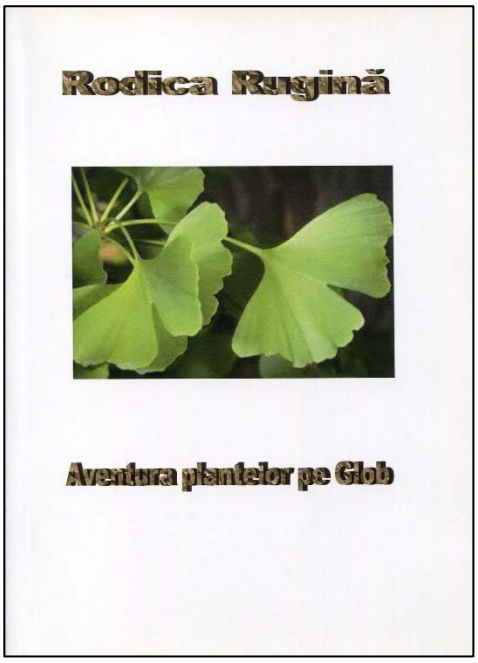
Most part of the 35 years of professional activity of the researcher Rodica Rugină consisted in the coordination of the Taxonomic Section of the Botanical Garden of Iaşi, a defining element among all the sections of the institution; hence the great responsibility in conserving and enriching the collections of characteristic plants. In order to face the inherent difficulties, she passionately and professionally involved in all activities specific to "ex situ" conservation: collecting plant material directly from spontaneous habitats, obtaining specimens from species as diverse as possible through appropriate horticultural techniques, from similar units in country and abroad, determination and redetermination of species in collections, collection of plants for "Flora Exsiccata of Moldova and Dobrogea". She paid special attention to the rare plants collected from different parts of the country and made efforts to adapt and even naturalize them. Field work was also accompanied by laboratory work, most cultivated species being studied under a research contract conducted over a period of 5 years (*Studies of conservation of the genofond of rare and protected plants in Romania, their introduction and acclimatization in the Botanical Garden of Iaşi*).

All throughout her activity at the Botanical Garden she collaborated with the successive directors of the institution (Emilian Ţopa, Constantin Toma, Mandache Leocov and Mihai Mititiuc), in order to permanently enrich the diversity of species in the section she coordinated. She also strived herself to increase the interest of visitors by creating an open-air amphitheater, a small pond for aquatic plants and some fountains. To all this we must add her involvement in guiding both regular visitors and students from different academic fields (Biology, Pharmacy, Agronomy, Geography, etc.).

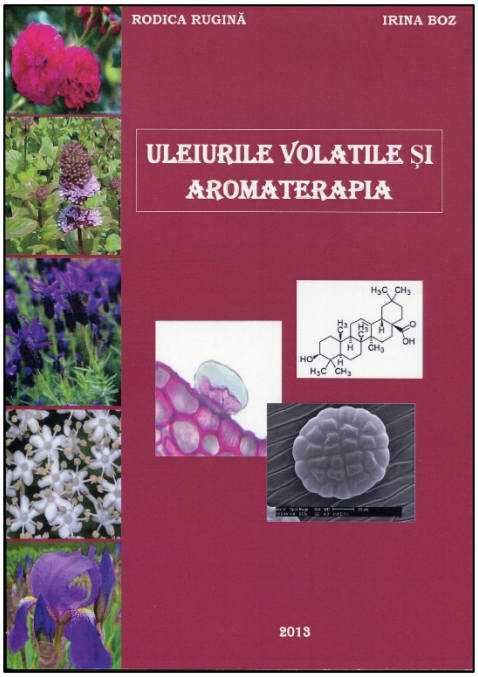
The decades dedicated to the cultivation and study of plants, which she deeply loves, were marked by the passion, seriousness, tenacity and critical spirit with which she approached each and every activity. For our younger colleagues she remains a model of professionalism.

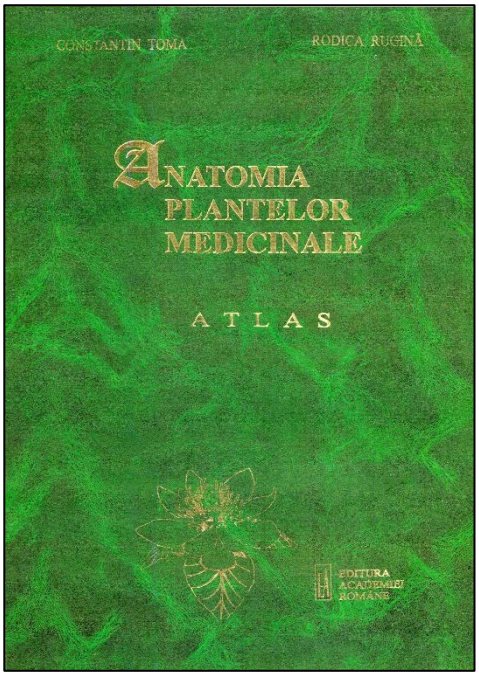
Her 80th anniversary is that special moment in which colleagues and all those who appreciate her activity can express their gratitude wishing her many happy returns of the day, good health and good spirits!

Lidia ADUMITRESEI, Camelia IFRIM
"Alexandru Ioan Cuza" University of Iaşi, "Anastasie Fătu" Botanic Garden


Rodica Ruginã
Aventura plantelor pe Glob


Rodica RUGINĂ
Mihai MITITIUC
PLANTE OCROTITE DIN ROMÂNIA
EDITURA UNIVERSITĂȚII „ALEXANDRU IOAN CUZA" IAȘI


RODICA RUGINĂ
IRINA BOZ
ULEIURILE VOLATILE ȘI AROMATERAPIA
2013


CONSTANTIN TOMA
RODICA RUGINĂ
ANATOMIA PLANTELOR MEDICINALE
ATLAS
EDITURA ACADEMIEI ROMÂNE

Academician Constantin TOMA (1935–2020)

Academician Constantin TOMA passed away unexpectedly, on September 8, after a life dedicated to science and education, to students and disciples.

Born on November 19, 1935, in the village of Gugești, Vaslui County, he attended the Primary School in the village of Gugești and in Huși (1943-1947), the gymnasium in Huși and Gugești (1947-1950), the Theoretical High School "Cuza-Vodă" in Huși (1950-1953) and the Faculty of Natural Sciences within the "Alexandru Ioan Cuza" University from Iași (1953-1958).

After graduating the university studies, he became a teacher at the Faculty of Biology within the "Alexandru Ioan Cuza" University of Iași, achieving through competition, due to his tenacity and hard work, the academic functions of preparator (1958-1959), assistant professor (1959-1966), lecturer (1966-1972), associate professor (1973-1977), full professor (1978-2005), *Professor Emeritus* (2005), teaching courses and coordinating practical research activities in the field of plant morphology and anatomy.

The scientific activity was mainly carried out in the field of plant biology (1958-2020). It was materialized in 471 scientific articles (of which 55 abroad) and 35 books published by prestigious publishers in the country and abroad, to which are added 100 articles of science popularization, 106 articles on the history of biology, 43 book reviews, and 20 chapters in collective volumes.

He researched from a morphological and histo-anatomical perspective 30 ecotypes, 150 varieties, and 553 species belonging to 170 genera and 55 botanical families, most of them being studied for the first time in our country.

Exceptional phyto-morphologist and anatomist, in the six decades of activity, the Academician Constantin Toma developed and modernized at the Faculty of Biology in Iași the most valuable school of plant morpho-anatomy in the country, widely recognized for outstanding performances nationally and internationally. With pedagogical tact and a lot of professional dedication, he collaborated with his disciples of all ages, and constantly updated the requirements of this scientific field with multiple interdisciplinary approaches. He carried out botanical studies in the field and specialization internships in Austria, Belgium, Bulgaria, Czech Republic, France, Germany, Italy, Great Britain, Republic of Moldova, Russia, Slovakia, Spain, Turkey, Ukraine, Hungary.

Since 1988 he became PhD coordinator in the scientific field of Botany, and since 2006, the executive director of the Doctoral School, contributing essentially to the scientific coordination and public defense of 35 doctoral theses.

Thus, the Professor contributed to the training of researchers working at various institutions and universities in Iaşi (Botanical Garden, Institute of Biological Research,

Anthropological Research Center "Olga Necrasov" within the Romanian Academy (Iaşi Branch), "Gr. T. Popa" University of Medicine and Pharmacy, "Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine), from the country (Bucharest, Arad, Bacău, Oradea, Piteşti, Timişoara, Târgu Mureş; museums and cultural associations from Bacău, Comăneşti, Piatra Neamţ, Vaslui) and from abroad (Republic of Moldova, Belgium, Italy, France, Spain).

During 1970-2001 he held various management positions: director of the Botanical Garden (1970-1973), vice dean (1975-1976) and dean (1989-1990 and 1996-2001) of the Faculty of Biology, head of department (1977-1985, 1990-1996), scientific secretary of the Senate of the "Alexandru Ioan Cuza" University (1990-1992), director of the Institute of Biological Research in Iaşi (1986-1990), corresponding member of the Romanian Academy (since 1991), honorary member of the Academy of Sciences of the Republic of Moldova (since 2011), full member of the Romanian Academy (since 2012), scientific secretary of the Romanian Academy - Iaşi Branch (2000-2001), vice president and honorary president of the National Society of Biological Sciences (since 2007), chairman of the Subcommittee on Natural Monuments of Moldova (since 1991), member of the National Council for Academic Evaluation and Accreditation (1994-2005), member of the Biology Commission of the National Council for Attestation of University Degrees, Diplomas and Certificates (1994-2005), member in the Council of the Publishing House of the Romanian Academy (1996-2003).

Distinctions with which he was awarded: highlighted professor (1982), the Order "Merit for Education in the rank of Knight" (2004), Professor Emeritus of the "Alexandru Ioan Cuza" University from Iaşi (2005), Awards of the Romanian Academy – "Emanoil Teodorescu" (1978, 2000, 2002) and "Emil Racoviță" (2016), Doctor Honoris Causa of the Universities of Arad, Bacău and Oradea, honorary citizen of Gugeşti village (2011) and of Iaşi municipality (2019).

The respect for the work of predecessors and contemporaries places the Academician Constantin Toma in the long line of scientists who transmitted, to the Romanian people, culture, dignity and a great responsibility to preserve, enhance and pass on the values of science and biological education.

Through his hard work, he proved that he can overcome everything, through his becoming, he honored, with respect, the memory of his parents and teachers, and through his work, he will remain forever in the memory of those who valued, respected and appreciated him!

He waited with dignity and serenity for the moment of departing from this world, to which he gave all he had best, leaving in a letter to his collaborators the following testament words: "No one should cry; I lived long and beautiful. I was appreciated in time for everything I did. I am proud of my students ..."

Lăcrămioara IVĂNESCU
"Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology
Cătălin TĂNASE
"Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology
Constantin MARDARI
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iaşi

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Professor Dr. Mihai MITITIUC (1937–2020) – successor of mycological research in Iași

On January 3rd, 2020, Professor Mihai MITITIUC, scientific personality of the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași, passed away.

In over 40 years of academic activity he proved an outstanding tenacity, responsibility, and a special organizational capacity. His remarkable scientific contributions in the study of fungi, recommend him as the successor of prestigious names belonging to the Mycology School of Iași: Ion C. Constantinescu, Alexandru Popovici, Constantin Sandu-Ville, Alexandru Lazăr, Constantin Dobrescu, Adrian Volcinschi, Mircea Hatman and Mihai Toma.

Professor Mihai Mititiuc was born on March 16th, 1937, in Sinăuții de Jos village (now part of Mihăileni commune), Botoșani county, in a family of respected countrymen, with great love for the land.

In 1944, he started the primary school in his village, and after this he attended the Medium School of Boys in Dorohoi (now "Grigore Ghica" Highschool), which he graduated in 1955. After the baccalaureate he enrolled at the Faculty of Natural Sciences-Geography of "Alexandru Ioan Cuza" University of Iași, which he graduated as valedictorian in 1960.

Starting with 1964 he was accepted for doctoral studies at the Faculty of Biology, University of Bucharest, having the privilege to collaborate with a great personality of the Romanian mycology – professor dr. docent Olga Săvulescu. His PhD thesis – *Contribution to the knowledge of micromycetes and macromycetes from Ponoare and Frumoasa natural reserves* (*Suceava county*) held in 1968, represents a reference work in the field, the results of his research correlating aspects regarding the specific taxonomical diversity of the plant communities of the two reserves with the ecology of several species of micro- and macromycetes. The doctoral committee of his thesis included noteworthy personalities from the field: professor dr. docent Constantin Sadu-Ville (corresponding member of the Romanian Academy), professor dr. docent Traian Ștefureac and dr. docent Vera Bontea, who all appreciated with high grades professor Mihai Mititiuc's research.

His thesis includes important taxonomic, ecological and chorological data regarding 460 species of fungi: 430 species of micromycetes on 305 plant hosts and 30 species of macromycetes. Also he described for the first time in Romania 39 new species of micromycetes and 37 known species and mentioned on 86 new plant hosts.

The species *Ascochyta petrakii* Sandu-Ville et Mititiuc, *Phoma camelinae* Sandu-Ville et Mititiuc identified on plant hosts from Frumoasa Reserve and *Sphaerotheca săvulescui* Sandu-Ville, *Phyllosticta salviae* Sandu-Ville et Mititiuc and *Rhabdospora*

anthemidis Sandu-Ville et Mititiuc identified on host plants from Ponoare Reserve are considered new for science.

The results of his microstationary research revealed the influence of humidity on the combinations fungi-plant host: *Septoria tormentillaea–Potentilla erecta*, *Capnodium salicinum–Filipendula ulmaria*, *Cytoplacosphaeria rimosa* and *Napicladium arundinaceum–Phragmites communis.* He also mentions the presence and frequency of 30 species of micromycetes in the following phytocoenoses: *Festucetum vallesiacae*, *Stipetum capillatae* și *Agropyretum repentis*.

These results were published in 12 scientific articles, published between 1965 and 1972 in national and international journals, such as: *Sydowia*, *Boletim da Sociedade Broteriana*, *Nowa Hedwigia*, *Mycopathologia et Mycologia applicata*, and *Feddes Repertorium*. His collaborations were also materialized in over 250 scientific articles published in specialized journals belonging to the Romanian Academy, Romanian universities, and volumes of national and international conferences. These papers include relevant research regarding the taxonomy and ecology of micro- and macromycetes, structural, physiological, and biochemical modifications induced by pathogenic fungi.

The research regarding the taxonomy of fungi, included in international data bases, specialized journals and conference volumes reveal exceptionally results that led to the discovery of 35 species of micromycetes new for science.

Professor Mihai Mititiuc begun his university career in 1960, by preparing and teaching the laboratories for the *Plant morphology and anatomy* course. Starting with 1962, as university assistant, he was also responsible for the laboratories of several other courses, such as: *Biology of plant pathogens*, *Phytopathology*, *Plants Protections* and Biogeography and for the students enrolled in the Biology and Geography departments. He also prepared a *Botany* course dedicated to foreign students in the preparatory year, for the Faculty of Pharmacy. His teaching capabilities were appreciated both by colleagues and students, giving him the opportunity to occupy, by contest, all the didactic degrees, up to the rank of university professor.

Consistent with the principles, proving abilities, initiatives and new approaches, with unmistakable in style and collaboration manner, he contributes, as professor, to the formation of numerous generations of biologists, who he accompanied in Carpathians forests and meadow, in the Danube Delta or in several reserves in Dobrogea, cultivating the passion and dedication towards the discovery and preservation of nature.

For the good development of student's field works he elaborated as only author or in collaboration, 11 books, 6 chapters in monographies and 4 university manuals. These volumes were dedicated both to students, PhD candidates, and for specialists in the field of natural sciences, forestry, agriculture, human and veterinary medicine and contained critically analyzed information regarding the state of the art in the specific field, completed with personal mycological research regarding the structure, function, taxonomy and ecology of fungi and vascular plants. The scientific content is very well documented and argued, including new data concerning different categories of fungal spores, fungal reproduction and phylogeny, the role of fungi as bioindicators for different substrates and habitats, and their importance in the ecosystem's stability.

Professor Mihai Mititiuc was also involved in the formation of young specialists in the field of mycology and phytopathology and starting with 1991 he was assigned PhD coordinator for the fundamental domain of *Natural Sciences*, *Biology* domain, *Botany* specialization, successfully coordinating 22 PhD thesis.

For the good development of the didactic and research activities he organized in 1993 the Mycology and Phytopathology Laboratory within the Vegetal Biology Department.

His special professional training and his exceptionally organizational capacity recommend him as director of the Botanical Garden (1990-2007). Under his management the specific infrastructure of this institution was accordingly developed: three new greenhouses, utility spaces and a thermal power plant were built, together with the reorganization of the scientific sections. In a period with numerous economical and social problems he managed to keep the integrity of the largest botanical garden in Romania, being permanently helped by the management of "Alexandru Ioan Cuza" University of Iași.

He always supported the publication of the Botanical Garden of Iași, another constant preoccupation being collecting fruits and seeds for the *Index Seminum et Sporarum*, biological material belonging to common or rare plant species, which was offered in exchange to other similar institution from Romania or abroad, or entered in the inventory of the Botanical Garden of Iași. Since working on his PhD thesis in the Ponoare-Bosanci and Frumoasa-Moara (Suceava county) Nature Reserves, Professor Mihai Mititiuc contributed to the diversification of the herbarium collections belonging to the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași, where he deposited no less than 2731 herbarium sheets: 2658 sheets with micromycetes, 20 sheets with macromycetes and 53 sheets with vascular plants. He had an important contribution in elaborating several volumes within the series *Flora Moldaviae et Dobrogeae Exiccata* edited by the Botanical Garden of Iași: *Centuria II* (1970), *Centuria III* (1972), *Centuria IV* (1974), *Centuria V* (1981), *Centuria VIII* (1994) and *Centuria IX* (2005), for the last two being co-editor, along with biologists Ion Sârbu and Adrian Oprea.

Moreover, he decisively contributed to the enrichment of plant collections from the Botanical Garden of Iași, he initiated collaborations with similar national and international institutions and supported the organization of several floral exhibitions and scientific events such as the anniversary symposiums celebrating 140 years (1996) and 150 years (2006) from the foundation of the first Romanian botanical garden, offering honorary medals and excellency diplomas to several personalities from the field of Botany and Mycology.

His organizational capacity, working strength and perseverance made him fit to manage non-governmental organizations (*Romanian Mycological Society* and *Association of the Botanical Gardens from Romania*), scientific societies and editorial boards of several journals: editor in chief of *Bulletin of "Anastasie Fătu" Botanical Garden of Iași*; member in the editorial board of *Scientific Annals of "Alexandru Ioan Cuza" of Iași, Vegetal Biology Series* and *Botanical Contributions of "Alexandru Borza" Botanical Garden of Cluj-Napoca*; member in the *National Academy Committee for granting PhD titles of Republic of Moldova*; member in the *Romanian Biological Sciences Society*; member in the *Association of Romanian Scientists*; member in the *"Ștefan Lupașcu" Foundation for Science*.

Professor Mihai Mititiuc coordinated for 12 years (1995-2007), as president, the activity of the *Romanian Mycological Society*. Through the projects and specific activities, he continued the initiatives of the founder of this society, Professor Mihai Toma. Moreover, he was involved in the organization of the 12th edition of the National Mycological Symposium and the 2nd National Biology Congress, held in Iași, in 1992.

His biographical data, the recognition of his scientific merits and also appreciation on the activities Professor Mihai Mititiuc supported and organized were mentioned by Ion

Popescu-Sireteanu (1994) in *Siretul, vatră de istorie și cultură românească* (*Siretul, Romanian history and culture hearth*), by Aurel Ardelean and collaborators (2000) in *Dicţionarul biologilor români* (*Dictionary of Romanian biologists*), by Petru Bejinariu (2003) in *Biologi de seamă din Bucovina* (Famous biologists from Bukovina), in the "Grigore Ghica" Highschool's from Dorohoi *Monography*, by Liviu Călcâi in the 3rd volume *Cu materialul clientului* (*With the client's material*).

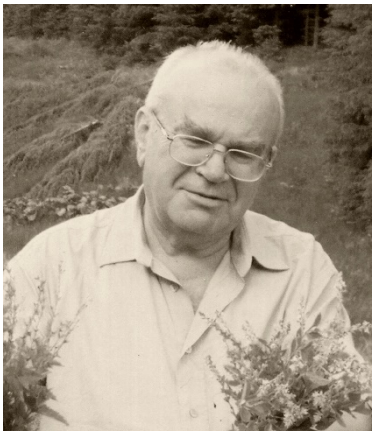
Professor Mihai Mititiuc constantly showed great initiatives and established successful collaboration, contributing to the formation of well-prepared disciples, who continue the tradition of mycological research in Iași and other national and international institutions.

Professor Mihai Mititiuc's personality will always last in the memory of his collaborators and students who valued and respected him.

May God light his way and soul for an eternal life in heaven!

Cătălin TĂNASE
"Alexandru Ioan Cuza" University of Iași, Faculty of Biology
Ana COJOCARIU, Cristiana Virginia PETRE
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iași

Professor Dr. Neculai ŞTEFAN (1943–2020)



In the middle of this summer (July 2020) one of the contemporary Romanian botanists, Professor dr. Neculai Ştefan, left this world for ever. Neculai Ştefan was connected profesionally with the universitary center in Iaşi, where he began his studies, and consolidated his career. Iaşi is the place where he founded his family, being permanently connected with his numerous friends, colleagues of department, students and graduates, etc.

He was born in Râmnicu Sărat (Buzău), in March, 8th, 1943, being the 3rd son of Dumitru and Dumitra Ştefan. He attended primary and secondary school in his native town, graduating them in 1960. Then, between 1960 and 1963 he studied in Buzău city, attending the courses at the Sanitary Technical School, and in 1963 he began his universitary studies, at Biology-Geography Faculty, having as his speciality Biology-Botany at the University "Alexandru Ioan Cuza" Iaşi.

After graduation in 1968 he began his instruction research at the Biological Research Center, which belongs to the Romanian Academy Iaşi Subsidiary.

In 1972, after he had finished his instruction research he continued his career as a Biologist in the same institution until 1977, when he was promoted as a scientific researcher I, taking all the exams in the scientific hierarchy.

In 1980 he took his scientific title as a Doctor in Biology, in Vegetal Biology Department, with his doctoral thesis "Research of Flora and Vegetation in Superior and Middle Basin of Râmnicu Sărat River", under the coordination of Professor Constantin Burduja.

In 1977 he participated at the competition for taking the lecturer degree at the Vegetal Biology Department and then, in 2001, he was promoted as a Professor at the same Faculty, until 2008, when he retired as a pensioner.

He taught the students, both in Biology and Geography, more lectures as: Systematical Botany (Cormobionta), Phytocoenology and the Romanian Vegetation, Nature Protection, Forestry Resources, Weeds and their Extirpation, The Theory and Practice of Durable Development and Forestry Resources, Principles of Ecology, Principles of Taxonomy, Medicinal and Aromatic Plants, General Botany and Geobotany.

He directed, yearly, laboratory practice and field works of the students in various locations, both at Systematical Botany and Phytocoenology.

His scientific activity, developed in 28 years as a researcher and then as a Professor, had as a result the publishing as a single author or in collaboration, of 214 original scientific articles in reviews, both in the country and abroad, 16 books and universitary teaching courses, to which are added 6 articles to popularize the science. These scientific works had the following principal directions: Systematical Botany (Cormobionta), Phytocoenology, Pratology, Herbology, Nature Protection, Ecology, The Study of the Drying Phenomenon of the Forests, The Influences of the Noxes upon the Vegetal Carpet, etc.

The Professor dr. Neculai Ştefan was connected more than affectively by the Romanian Danube Delta Biosphere Nature Reserve, where he had made numerous longer or shorter scientifical trips, and the scientific results were published in collaboration of more synthesis works as: "Explanation to the Vegetation Map of the Romanian Danube Delta Biosfere Reserve" (Flevobericht, Lelystand, Netherlands, 1994); "Reconstrucţie ecologică în Rezervaţia Biosferei Delta Dunării, Romania, Ostroavele Babina şi Cernovca" / "Renaturierung im Biosphären reservat Donau-Delta, Romänien) Romania / (Tulcea-Omwelstiftung WWF, Deutschland", 1997); "Delta Dunării - Rezervaţie a Biospherei" (Publishing House Dobrogea, 2006), etc.

In 1997 he participated at a workshop in Barcelona, within the International Project Eureed II, and in 1999 he was invited to the Research Station "Cocodrie" in Louisiana (USA), where he gave a lecture about floristical and phytocoenological biodiversity of the Danube Delta reeds and he made practical applications in "Barataria Preserve" and in Mississippi River Delta. He was invited to the 5th *Hieracium* workshop (Germany, 2000) and to the 6th *Hieracium* workshop (Austria and Germany, 2002).

In 2010, he was invited to the University of Fuzhou (China), within the Romanian-Chinese exchange project "Research on Landscape and Environment at Different Scales", focused on urban sustainabality scenarios, in collaboration with Fujian Normal University (2009-2010).

In his quality as a wellknown botanist, with a valuable experience along the years, he obtained and coordinated a number of over 40 scientifical research projects with dometic financing, as: "Research on Floristical and Phytoceonological Diversity in Obcinele Bucovinei" (2001); "Floristical and Phytocoenological Studies on National Park Cheile Bicazului-Lacul Roşu-Hăşmaşul Mare Massive" (2003); "Terrestrial and Aquatical Peri-urban Ecosystems in Ciric River Basin, in the North of Iaşi" (2007); "Studies for Preservation of Endemic Species *Andryala levitomentosa* in Natural Habitats" (2007); "Project *Nardus* (Inventariation of the Grasslands in Romania" – Partnership with the National Institute of Research-Development Danube Delta Tulcea (2007).

Thanks to his professional competence, Neculai Ştefan was coopted in more teams in order to realize scientifical programmes with international and national financing, as: the Project of the European Agency of Environment "CLC 2007 / GMES FTS Land Monitoring Romania"; Project PNCDI-2 "Strategies Agro-forestry for Valuation and Conservation of the Medicinal Plants Biodiversity in the Context of Rural Durable Development on the Mountain Valley of Bistriţa River" (2007); Project Nucleus-Biostar 06-400-401 (2006-2007); Project "Identification and Obtaining of Bioactive Phytocomplexes from Medicinal Species, for Making some Formulas of Nutritional Supplements used as Antitoxin and Hepatoprotectors"; Project PCCD-BIOTCH "Ecological Reconstructions by Micoremediation Proceedings of Degraded Soils of Mining Activities" (2007).

In his quality as a Professor he supervised over 60 papers of taking degrees of disertations, and he was a member in twelve committees for the title of Doctor in Biology.

Another aspect of his activity was the support given training to the teachers from the secondary schools, supervising more graduation papers of I degree from a scientific point of view and participating in committees of obtaining didactic degrees.

He was a member of more scientific societies, both in the country and abroad, as: Romanian Biological Society; Society of Phytocoenology in Romania; Amicale Internationale de Phytosociologie (France); International Association for Vegetation Science (IAVS); Society for Conservation Biology, etc.

His recognition as a specialist in Botany, both in Romania and abroad, had as a result to enlist his name in various catalogues, indexes, dictionaries of speciality, as: Experts

Register, first edition (1997), edited by the Romanian Academic Society; Membership List, IAVS (1998); Taxonomists Index of Romania, Publishing House "Pentru Viaţă" (1999); Romanian Biological Dictionary, vol. II, Publishing House of West University "Vasile Goldiş", Arad (2000).

All these achievements and many others which were not mentioned in this necrology contribute to the commemoration of Neculai Ştefan, not only as a Professor, but also especially as a man, and represents a necessary giving at the knowledge of the Botanical science development in Romania. Besides these, he contributed essentially to the training and educating of several series of students, master students or doctoral students who will carry on the traditions of the Romanian biology.

Regarding me, I knew him as a man in 1990's of the last century and I can tell, being very convinced, that he was an example for me from a professional and a human point of view. I remember piously how vehement he was in those times when he wanted to defend a colleague or even unknown people in front of injustice, unhonesty, hypocrisy or in front of certain arbitrary decisions of certain temporary persons or leaders of some public institutions.

He also remains a veritable portrait of a man who had an authentic respect for the predecessors in Biology, Geology, and Geography – a world which was close not only to him, but also to us from a professional point of view.

We, as well as the next generations, should pay our homage, piously and gratefully to Professor Neculai Ştefan.

Adrian OPREA
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iaşi

BOOK REVIEW

TOADER CHIFU, CONSTANTIN MARDARI, ***Diversitatea floristică şi fitosociologică a vegetaţiei din masivul Ceahlău* [*Floristic and phytosociological diversity of vegetation in Ceahlău massif*]**, 2020, RAO Distribuţie Publishing House, 204 p., ISBN 978-606-006-496-1.

The book *Flora and vegetation of Ceahlău massif* is part of the regional monographic researches, which have lately known a special intensity, research that is part to the wider study of biodiversity, with an important role in the economy of man and nature. Through this work, the authors make a contribution to the study of the flora and vegetation of one of the most famous mountainous areas in Romania and highlight the effort of the predecessors in the study of the extraordinary phytodiversity of this mountain massif.

The first part presents the flora of the Ceahlău massif together with a short history of botanical researches that highlights, starting from the first floristic mentions dating from the end of the 18th century, until now, the contributions of all researchers of the flora in this region. The vascular flora includes 1288 taxa, classified in 460 genera and 97 botanical families. Also, the authors emphasized the specifics of the flora in this mountain area. The endemic elements were highlighted, as well as numerous other species protected in Romania. Aspects related to species distribution depending on vegetation belts and other phytogeographical features of the territory of the Ceahlau massif were also presented. The second part was dedicated to the study of phytosociological diversity. After a short history of phytosociological studies and the presentation of the large vegetation units in Ceahlău massif, the syntaxonomic scheme including 56 coenotaxa within 18 vegetation classes was highlighted. For each association/sub-association were presented the distribution, the characteristic/differential species and a characterization of the floristic and phytocenological composition. Among all plant communities, the diversity of the meadows was highlighted.

This book addresses a topic of particular scientific importance in the field of biodiversity, presenting a broad synthesis of data known in the literature, completed by a valuable personal contribution. Due to the richness of the data contained in the main chapters, the paper is a meritorious and topical scientific achievement that comes to complete the research in the field of biodiversity.

Ciprian Constantin BÎRSAN
"Alexandru Ioan Cuza" University of Iaşi, "Anastasie Fătu" Botanic Garden
Cătălin TĂNASE
"Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology

BOOK REVIEW

COSTEL VÎNĂTORU, BIANCA MUȘAT, CAMELIA BRATU, ***Tratat de legumicultură specială* [*Special vegetable growing treaty*]**, 2019, ALPHA MDN Publishing House, Buzău, 886 p., ISBN 978-973-139-453-4.

Plants represent a unique category of organisms, capable of producing organic matter from water and mineral compounds, being the primary producers in many trophic chains. For thousands of years, people use plants as a main food resource, developing and perfecting growing protocols and methods in order to improve the quality and quantity of the vegetal products.

In Romania, Buzău County is considered to be the cradle of the Romanian vegetable growing, due to the special pedo-climatic conditions found here. To harness the valuable potential of this region, in 1957, the first Romanian Research Station for Growing Vegetables was established here. This excellency institution is a strategic unit, where specialists aim to improve and optimize the crop technologies for open fields and greenhouses, select and produce biological material for several vegetable and flower varieties, research in the fields of agrochemistry, physiology and biochemistry.

In this volume, the authors gathered a major part of their work experience regarding the growing technologies for many species of vegetables, fungi, aromatic and condimentary plants, information structured in 13 chapters, an Annex and selective references, eloquent for this field of research.

The first 10 chapters present the specific growing techniques for 135 species of plants, starting with the most common ones, widely cultivated nowadays, some old, traditional species and varieties that are now somehow forgotten, but which are very well adapted to our country's pedo-climatic conditions and also some new, acclimated species, of foreign origin, but nutritionally and medicinally valuable. These annual or perennial species are grouped according to the plant's organ (part) used for the mentioned purpose: **subterranean organs**: carrot (*Daucus carota* conv. *sativus*), parsnip (*Pastinaca sativa* conv. *hortensis*), celery (*Apium graveolens*), onion (*Allium cepa*), potato (*Solanum tuberosum*), radish (*Raphanus sativus*), yacón (*Smallanthus sonchifolius*), beet (Beta vulgaris), etc., **leaves**: salad (*Lactuca sativa*), arugula (*Eruca sativa*), endive (*Cichorium endivia*), Okinawa spinach (*Gynura bicolor*), amaranth (*Amaranthus* sp.), basil (*Ocimum basilicum*), mint (*Mentha* sp.), rosemary (*Rosmarinus officinalis*), etc., **fruit**: tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita pepo*), bitter melon (*Momordica charantia*), African cucumber (*Cucumis africanus*), pepino (*Solanum muricatum*), sponge gourd (*Luffa cylindrica*), etc. or **seeds**: beans (*Phaseolus vulgaris*), peas (*Pisum sativum*), broad bean (*Vicia faba*), chickpea (*Cicer arietinum*), sweet corn (*Zea mays* ssp. *saccharata*),

coriander (*Coriandrum sativum*), chia (*Salvia hispanica*), sesame (*Sesamum indicum*), etc. For every species, the authors offer readers important information regarding the plant's taxonomy, scientific and popular names, origin and their worldwide spreading, botanic description, alimentary and medicinal properties, other uses, growing technology (relation with the abiotic factors, types of cultures, sowing, specific works, controlling pests and diseases, harvesting).

Chapter 11 includes relevant information regarding the culture technology for several species of mushrooms (*Agaricus* sp., *Pleurotus* sp., *Lentinula edodes*, *Coprinus comatus*, *Stropharia rugosoanullata*, etc.) and chapter 12 is dedicated to plant species with eatable flowers, rich in nutrients and medicinally valuable compounds (*Agastache foeniculum*, *Bellis perennis*, *Brassica juncea*, *Borago officinalis*, *Calendula officinalis*, *Viola odorata*, *Tropaeolum majus*, etc.).

Chapter 13 presents the main diseases (viroses, bacteriosis and mycosis) and pests (nematods, mites and spiders, insects, snails, birds, mammals) that attack crops, affecting the quality of the vegetal products, together with methods for preventing and controlling them. An important subchapter presents the nutrient deficiencies in vegetables and their manifestations.

The written information is accompanied by a rich collection of original pictures and diagrams, meant to better illustrate the plant's morphology (general aspect, subterranean organs, stem, leaves, flowers, fruits, seeds), symptoms of diseases and pests attacks, nutrient deficiencies and field management.

The volume constitutes an important theoretical and practical tool dedicated both to professionals and amateurs, the culture technologies being perfected over the years by specialists from one of Romanians most influential Research Station for Growing Vegetables.

As the first author states, this book aims to "*enrich the growers, processors and consumers knowledge* […] *and everybody's who loves and practices one of the most noble and beautiful occupations – gardening, starting from the pot in the apartment and getting to the precision vegetable growing in the field or in the greenhouse*".

Cristiana Virginia PETRE,
Mihaela MIHALACHE
"Alexandru Ioan Cuza" University of Iaşi, "Anastasie Fătu" Botanic Garden
Cătălin TĂNASE
"Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology

JOURNAL OF PLANT DEVELOPMENT
GUIDE TO AUTHORS

AIMS AND SCOPE OF THE JOURNAL

Journal of Plant Development is the official scientific journal of the "Anastasie Fătu" Botanical Garden, which belongs to "Alexandru Ioan Cuza" University of IAŞI, ROMANIA. It was first published in 1979 (at that time as "Culegere de Studii şi Articole de Biologie"). The new series begins in 1993 under the name "Buletinul Grădinii Botanice Iaşi". From 2008 on, it has been published under its present name "**Journal of Plant Development**". It appears in one volume, with one or two issues per year.

Journal of Plant Development (JPD) is an international journal that acts as a medium for the exchange of ideas and provides publication (yearly) of articles in all areas of Plant Science and Botany (of all 'plant' groups in the traditional sense - including algae, cyanobacteria, fungi, myxomycetes). It covers topics in plant development field, as well as the plant ecology. The Journal also covers related fields such as: plant conservation, plant taxonomy, plant embryology, phytosociology, ecology, plant morpho-anatomy and histology, comparative and developmental morphology, physiology, ecophysiology, plant distribution, natural and artificial habitats, ornamental plants, pharmaceuticals uses of plants, plant molecular biology, plant cell, tissue and organ culture etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. All articles published in JPD are peer-reviewed.

TYPES OF MANUSCRIPTS AND LANGUAGE

The journal publishes original research articles, short communications and reviews in English. Journal of Plant Development also publishes book reviews and conference reports. Manuscripts may be of any length, but must be clearly and concisely written.

Three main *types* of manuscripts may be submitted:

Original research articles: should reports results of a substantial, completed and original work, and describe new and carefully confirmed findings. Experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short communications: are suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods or techniques. The style of main sections need not conform to that of original research articles. Short communications are 2 to 4 pages in length.

Review articles. Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Review articles are critical evaluations of material that has already been published. By organizing, integrating, and evaluating previously published material, the author considers the progress of current research toward clarifying a problem. A review article is a tutorial in that the author defines and clarifies a problem, summarizes previous investigations in order to inform about the state of current research, identifies relations, contradictions, gaps, and inconsistencies in the literature, suggests the next step or steps in solving the problem. Reviews should be concise and no longer than 14-16 printed pages. Reviews are also peer-reviewed.

OPEN ACCESS POLICY

Journal of Plant Development publishes fully open access articles, which means that all articles are available on the internet to all users immediately upon publication. Non-commercial use and distribution in any medium is permitted, without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of open access.

REVIEWING POLICY

All contributions are subject to a double-blind reviewing process. Acceptance of papers is supervised by an international Editorial Board. Manuscripts considered suitable for peer review by the Editorial Board are sent to at least two referees (members of the Board or external). The journal gives referees a target of four weeks for the return of their reports. The review process takes between three weeks and two months.

PROOFS AND REPRINTS

Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage. Because JPD will be published freely online to attract a wide audience, authors will have free electronic access to the full text (in both HTML and PDF) of the article. Authors can freely download the PDF file from which they can print unlimited copies of their articles.

SUBMISSION

Manuscripts should be submitted electronically by sending a message to gbot.is@uaic.ro or ana.cojocariu@uaic.ro. The message should include:

(1) a cover letter, that should include the corresponding author's full name, address and telephone/fax numbers and should be in an e-mail message sent to the Editor. A Cover Letter is to be made upon submission, sending a revision or re-submission.

(2) a text file with the entire text, as an attachment, whose name should begin with the first author's surname.

(3) additional files for figures and tables.

Submission of a paper implies that it has not been published before (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors, and that, if accepted, will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher.

JPD Formatting and template: http://www.plant-journal.uaic.ro/instructions.htm

Journal of Plant Development have its own specific formatting. This defines how an article will look when it is published online or in print. In order to format your article ready for submission to JPD, **Word Template** are available on the website, ready for you to download and apply to your document. Read the instructions for the author/s, download and save the word template file to your computer and apply these styles to your paper as appropriate.

Authors should consult the **checklists** given here on **how to prepare the files**. Authors are expected to have their papers well checked for content and correctness in

presentation of text and illustrations. Manuscripts not conforming to the guidelines will be returned to the author until satisfactory files are provided.

The corresponding author receives by e-mail an acknowledgment of receipt of the manuscript, mentioning the communicating editor and a manuscript reference number (Article ID). The manuscript number will be mailed to the corresponding author same day or within 72 hours. If you do not receive an acknowledgement you should inquire to be sure it was received.

Details on types of contributions

1. Original research articles

The papers will be published only in a foreign language (English), structured as follows: title, authors, affiliation of the authors, abstract, keywords, introduction, material and method, results & discussions, conclusions, acknowledgements, references, tables, figure captions.

Title should be a brief phrase describing the contents of the paper.

Authors names would not be abbreviated, capitals for surname (family name) and no capitals for first name (except initial letter). Each author name would be accompanied by a complete address, as a footnote on the first page. The affiliation should be provided in the following order: university (institution) name; faculty/department name; number and street name; city; country and email address. One of the authors should be designated as the corresponding author.

Abstract should be concise informative and completely self-explanatory, briefly present the topic, state the purpose of the research, indicate significant data, and point out major findings and conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list. Non-standard or uncommon abbreviations should be avoided but, if essential, they should be defined at their first mention in the abstract itself.

Key Words: five to seven words, the most important ones, in alphabetical order, after someone could discover your paper on the internet engines. Key words should not repeat the title of the manuscript.

The **Introduction** should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines. The introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The **Discussion** should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the

paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

Conclusions should spell out the major findings of the work and may include some explanation of the significance of these conclusions and what further research should be done. Authors should include a general interpretation of the results in the context of current evidence, and not restricted to that which supports the findings of the present study.

Notes on contributors in maximum 65 words, provide short biographical notes on all contributors.

The **Acknowledgments** of people, grants, funds, etc. should be brief. People who contributed to the work but do not fit criteria for authorship should be listed in the Acknowledgments, along with their contributions. It is the authors' responsibility to ensure that anyone named in the acknowledgments agrees to being so named.

References should be listed at the end of the paper in alphabetical order. In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text. Authors are fully responsible for the accuracy of the references.

2. Short communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. Short Communications follow the same format as for the original research papers, with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion sections should be combined into a single section.

3. Review articles

Review articles are critical evaluations of material that has already been published. By organizing, integrating, and evaluating previously published material, the author considers the progress of current research toward clarifying a problem. Reviews should be concise and no longer than 14-16 printed pages. Reviews are also peer-reviewed.

4. Book reviews and conference reports

These types of contributions would not exceed an A4 format page.

5. Special Issues

Proposals for Special Issues of full research papers that focus on a specific topic or theme will be considered.

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☐	**E-mail address** of the corresponding author.
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☐	A list of at least five to maximum seven **Key words**, in alphabetical order, that should not repeat the title of the manuscript.
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	☐	All references cited in text (also when exclusively used in the bibliographies of taxa in a taxonomic treatment) are provided in the References section and vice versa.
	☐	The references are arranged alphabetically based on the last name of the first or sole author. Where the same sole author or same first author has two or more papers listed, these papers are grouped chronologically. Where such an author has more than one paper in the same year, these are ordered with single authored papers first followed by two-author papers (ordered first alphabetically based on the second author's surname, then by year), and then any three-or-more-author papers (in year order only). Letters 'a', 'b', 'c', etc., are added to the date of papers with the same authorship and year.
	☐	References conform to one of the following styles according to the type of publication:
		☐ **Papers in periodicals**: the name of the author(s) would be given in capital letters. The Christian name(s) would be abbreviated. Before the last but one and the last author you must insert the sign "&". In the reference list you must mention all the authors of a certain paper. The year of a paper publication is put after the author (s). Title should be fully written. Between the year and the title we recommend to be inserted a point sign. Each periodical name is to be written in italics. A certain volume must be given in bolds. After it is placed the number of the periodical, inserted between the round brackets; next to it would be inserted the page numbers of the paper. BOSCAIU N. 2001. Ecotonul si importanta functiilor sale ecoprotective. *Ocrotirea Naturii.* **42**: 13-21. MEHREGAN I. & KADEREIT J. W. 2008. Taxonomic revision of *Cousinia* sect. *Cynaroideae* (*Asteraceae*, *Cardueae*). *Willdenowia.* **38**(2): 293-362.
		☐ ***Books*:** title should be fully written. The title of a book is written in italics. After the title, is placed the name of the town, the publishing house and the page numbers.

		BOSCAIU N. 1971. *Flora si vegetatia Muntilor Tarcu, Godeanu si Cernei*. Bucuresti: Edit. Acad. Române, 494 pp. HILLIER J. & COOMBES A. 2004. *The Hillier Manual of Trees & Shrubs*. Newton Abbot, Devon, England: David & Charles, 512 pp.
	☐	**Chapters in books:** author(s), year, title, pages, a point sign, followed by "In": author (s) of the book, city, publishing house, number of pages. TUTIN T. G. 1996. *Helleborus* L. pp. 249-251. In: TUTIN T. G. & al. (eds). *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, XLVI, 581 pp.
	☐	Article only available on-line with digital objet identifier (DOI): SONKÉ B., DJUIKOUO K. M. N. & ROBBRECHT E. 2008. *Calycosiphonia pentamera* sp. nov. (afrotropical Rubiaceae) from the 'Lower Guinea' area. *Nordic Journal of Botany*. http://doi.org/10.1111/j.0107-055X.2007.00141.x
	☐	Thesis: NTORE S. 2004. Contribution à la connaissance systématique du genre afrotropical *Pauridiantha* (Rubiaceae). PhD Thesis, K. U. Leuven, Leuven, Belgium.

CHECKLIST FOR *ILLUSTRATIONS*

☐	Photographs, drawings, maps, graphs, charts, plates and diagrams are all treated as 'Figures' and should be numbered consecutively in accordance with their appearance in the text.
☐	The mentions at the drawings, figures, pictures and tables will be placed inside the round brackets – for instance (Figure 2), (Table 2).
☐	All illustrations should be clearly marked with the figure number and the author's name.
☐	Figures composed of several smaller figures are treated as one figure and the subfigures are labeled with capital letters (A, B, C, D, etc.). Scientific names in captions are not followed by authors.
☐	Each table has a complete caption at the top and is Table 1, Table 2, etc. according to the order in which they are first mentioned in the text.
☐	All the schemes, drawings, electron micrographs etc. would be accompanied by a scale bar.
☐	Line diagrams are black and white. Use of colour in line diagrams is acceptable where this enhances clarity significantly.
☐	Related graphics are grouped into a single figure whenever possible. Each part of a grouped figure is labelled A, B, C, etc. and not treated as separate figures. Labels are preferably placed in the upper left or lower left corner of the picture using.
☐	Colour images are used where they enhance significantly the clarity of the scientific information and must be very clear, being accompanied by the explanations.
☐	The diagrams should be made in Excel; pictures, ink drawings must be saved in JPG, JPEG, or BMP format, having a good resolution.
☐	If figures or tables are not imported into your text, these would be placed on separate sheets, with indications of their inserting place in the text.
☐	Table text is typed using Times New Roman. Capitals are avoided within table cells (exceptions: first letter of the heading of row or column, names of taxa requiring a capital, and abbreviations e.g. pH).
☐	Tables must be cell-based. Do not provide tables as graphic objects.
☐	Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

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