PHARMACOGNOSY OF *Rauvolfia serpentina* (L.) Benth. ex Kruz IN THE ECOLOGICAL CONDITION OF DARJEELING DISTRICT OF WEST BENGAL

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This is to certify that research work embodied in the thesis "Pharmacognosy of *Rauvolfia serpentina* (L.) Benth. ex Kruz in the ecological condition of Darjeeling District of West Bengal", has been carried out by Smt. Debashri Paul, M. Sc., M. Phil. under my guidance.

She has fulfilled the requirements relating to the nature and period of research. It is also certified that the thesis incorporates the results of original investigation made by Smt. Debashri Paul in Medical Botany and Pharmacognosy Research Laboratory, Department of Botany, North Bengal University under my guidance and supervision. The thesis is now being submitted for the fulfilment of her Ph. D. degree in Science (Botany) under the University of North Bengal. It has not been submitted previously for any degree whatsoever.

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CONTENTS

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ABBREVIATIONS

AR	=	Analytical reagent.
BA	=	6 – benzyl adenine.
°C	=	Celsius.
$CaCl_2$	=	Calcium chloride.
$Ca(OH)_2$	=	Calcium hydroxide.
CH	=	Casein hydrolysate.
cm	=	Centimeter.
2,4-D	=	2,4-Dichlorophenexy acetic acid.
DAT	=	Days after transplanting.
df	=	Degrees of freedom.
EtOH	=	Ethyl alcohol
$FeSO_4$	=	Ferrous sulphate.
g	=	Gram.
GA_3	=	Gibberellic acid – 3
h	=	Hour.
HCl	=	Hydrochloric acid.
$HgCl_2$	=	Mercuric chloride.
H_2SO_4	=	Sulphuric acid.
IAA	=	Indole acetic acid.
IR	=	Infrared.
kg	=	Kilogram.
Kn	=	Kinetin.

KOH	=	Potassium hydroxide.
l	=	Litre.
λ	=	Lamda
LAR	=	Leaf area ratio.
LSD	=	Least significant difference.
LWR	=	Leaf weight ratio.
M	=	Molar.
MeOH	=	Methyl alcohol.
mg	=	Milligram.
max	=	Maximum.
min	=	Minute.
ml	=	Millilitre.
mμ	=	Millimicron.
mol	=	Molecular.
m.p.	=	Melting point.
MS	=	Murashige and Skoog medium.
μg	=	Microgram.
N	=	Normal.
NH	=	Secondary amine
NAA	=	Napthalene acetic acid.
NAR	=	Net assimilation rate.
NaOH	=	Sodium hydroxide.
nm	=	Nanometer.
NS	=	Non-significant.

O.D.	=	Optical density.
pH	=	Potential for hydrogen.
ppm	=	Parts per million.
Rf	=	Relative flow.
RGR	=	Relative growth rate.
RLGR	=	Relative leaf growth rate.
RLWC	=	Relative leaf water content.
S.E.	=	Standard error of mean.
SLA	=	Specific leaf area.
TLC	=	Thin layer chromatograph.
UV	=	Ultra violet.
Wt.	=	Weight.

GENERAL INTRODUCTION

Plants have for centuries been an important source of precursors and products used in a variety of industries, including those of pharmaceuticals, food. cosmetics and agrochemicals. Use of plants as drugs goes back to prehistoric times. The old Egyptians, Babylonians, Greeks, Roman, Chinese and Indians, all developed their characteristic materia medica. It is a remarkable circumstance that, alongside of modern medicine, the mediaeval Graeco-Arab system known as 'Unani Tibbi' (Ionian Medicine) and the ancient healing system of Ayurveda continue to hold the field in the Indian subcontinent, catering to the needs of a large majority of the population.

In the 1920's a concerted effort was made at the School of Tropical Medicine in Calcutta, towards the pharmacological investigation of the active constituents and isolation of a whole series of plant drugs. Subsequently, as a more purposeful approach from the point of view of the indigenous systems of medicine, a drug research institute was established at the Ayurvedic and Unani Tibbi College in Delhi, on the initiative of late physician and statesman Hakim Ajmal Khan. The very first drug that came up for investigation at this institute was *Rauvolfia serpentina*, which was systematically used over a long period by the late Hakim Ajmal Khan in the treatment of a variety of mental ailments.

The extraordinary medicinal properties of *R. serpentina* have brought this Indian drug plant to the attention of the medical world. The alkaloids obtained from its roots have the property of lowering blood pressure and are, consequently, widely used for a long time in the treatment of hypertension. With the help of clinical trial Rath *et al.* (1999) have observed similar effect of ayurvedic preparation containing *R. serpentina* to that of Atenonol 50. The drug is widely used as a sedative, hypotensive, tranquillizer in insanity and a host of other mental

ailments (Akram *et al.*, 1993; Roja and Heble, 1996). Its antirhythmic property was observed by Vollosovich *et al.* (1978). Very recently Pravathi Devi (2000) mentioned that the plant can be used to prevent menopausal problems and helps to keep the woman as feminine forever.

The *Rauvolfia* genus of Apocynaceae consists of more than 130 species (Woodson *et al.*, 1957), of which *R. serpentina* was the most important one. The name was assigned by the French botanist Plumeir in honour of the 16th century German physician and botanist Leonhart Rauwolf, who travelled extensively in the Middle East searching for plants with medicinal usefulness. Later the name of the genus *Rauwolfia* has been changed to *Rauvolfia* on the basis of a rule of Botanical Nomenclature.

R. serpentina is a small erect woody shrub, usually less than a meter in height and which bears attractive white or pink flowers. It flourishes in the moist deciduous forests of India, usually between sea level and 120 m. It grows wild along the Western and Eastern Ghats, in the lower hills of the Gangetic plain, and on the lower Himalayan slopes from Simba to Assam (Santapau, 1956; Bal, 1956; Kaul, 1956). The root system consists of permanent tuberous soft tap root up to 6 cm in diameter when fresh and having a corky bark with longitudinal fissures. In North Bengal once the plant was mentioned by J. D. Hooker as dominant species in Terai and Dooars region but from survey of the plant appears to be an endangered species as not a single plant of this species is available in wild condition (Nair *et al.*, 1991).

In spite of the wide reputation of *Rauvolfia* extracts in folk medicine, the curiosity of chemists was not attracted to their investigation until 1931. Greshoff in 1890 noted the presence of alkaloids in *R. canescens* L., but no further work was reported until 1931 when from the roots of *R. serpentina*, obtained from the Bihar province of India, Siddiqui and Siddiqui isolated a series of crystalline bases, namely, ajmaline, ajmalinine, ajmalicine, serpentine and serpentinine.

taking advantage of the differences in their basic strength on the one hand, and the solubility of their hydrochlorides on the other. Later in 1939, working on the roots and root bark of the plant, obtained from the more temperate climate of the Dun Valley, Siddiqui reported the isolation of two isomers of ajmaline, namely isoajmaline and neoajmaline, a base melting at 220°C and from the neutral fraction of the alcoholic extract, a white crystalline alkaloid melting at 234°C, with the yield of 0.1% on dry weight basis. From this last mention base and its mother liquors it was subsequently possible through repeated fractional crystallisations from methanol and acetone to isolate a crystalline substance, which melted at 270–273°C, and yielded on mild hydrolysis an acid and a base. These findings, however, were not reported, awaiting further work on the problem which had to be held over during the war and its aftermath.

In 1952, Swiss chemists Muller *et al.* reported the isolation of this base under the name, reserpine, from the so-called "oleoresin" fraction which actually corresponds to the 'neutral fraction' referred to above. Reserpine soon proved to have a very important therapeutic value and during the ensuing five years an intensive search was made for alternate sources of this alkaloid. By the end of 1956, the constituents of more than 20 *Rauwolfia* spp have been examined, most of which were shown to contain reserpine. In addition, a total of approximately 40 well authenticated alkaloids had been isolated, together with several less known ones, which have not yet been fully characterized.

It was not until 1815 that the term pharmacognosy was introduced by C. A. Seydler, a medical student in Halle, Germany. The name is termed out of two Greek words "pharmacon", the drug and "gnosis"—knowledge. The most comprehensive idea of the scope of pharmacognosy was presented by Fluckiger—who stated that it is the simultaneous application of various scientific disciplenes with the objective of acquiring knowledge of drugs from every point of view (Tyler *et al.*, 1976). In a restricted sense the definition of pharmacognosy implies

the study of plants yielding natural product having bioactivity from botanical and chemical point of view.

The cultivation of *R. serpentina*, which remains the best source of reserpine, has now become of highest importance, since the unrestricted harvesting of the plants soon threatened to exhaust the supplies available in India. This led the Indian government to encourage and foster its cultivation on a large scale.

In conventional method, the propagation of *R. serpentina* is done by seeds, root cuttings, stem cuttings and root stumps. It is usually propagated by seeds. The percentage of germination of seed is reported to be 10 to 50% (Badhwar *et al.*, 1956a; Hedayatullah, 1959; Nayar, 1956; Santapau, 1956). Due to poor and erratic germination of seeds and as such the collection of seeds from wild sources is both laborious and costly, vegetative propagation has been advocated for raising plantations. Badhwar *et al.* (1956b) compared the different methods of propagation of *R. serpentina* and their effect on root production.

A few reports indicate that some growth physiological and agronomical studies have been carried out with *R. serpentina*. Nandi and Chatterjee (1975) studied the effect of NPK fertilizers singly or in combination on growth and alkaloid formation in *R. serpentina* at high altitude of Darjeeling Himalayas. Their results revealed that high level of P only augmented the laminar area, whereas N when applied both singly or in combination, decreased it. Single application of N and K could not increase the total alkaloid contents. Maximum alkaloid synthesis was noticed in combination of N, P and K. Sahu (1970 a, b) and Maheswari *et al.* (1988) also studied the effect of fertilizers on growth, root and alkaloid yield of *R. serpentina* and reported similar observations. Maurya *et al.* (1999) observed that 60 kg N/ha was suitable for higher root yield of *R. serpentina* in Bihar, India.

Less information is available on irrigation scheduling of *R. serpentina*. Maheswari *et al.* (1991) observed that dry root and alkaloid yields and water use efficiency of *R. serpentina* increased up to an IW:CPE of 0.75. The alkaloid content was not affected by irrigation schedules.

Removal of flowering branches and cutting of flower buds have been recorded in the literature as the means of increasing the root yield along with the intensive formation of the secondary plant products. Biswas (1969, 70, 71, 73) studied the changes in the alkaloid contents of *R. serpentina* under defloration and nitrogen treatments. Complete defloration resulted in large increase of root weight. An appreciable increase in the total alkaloidal contents of the moisture free root was also observed.

Saini and Mukherjee (1970) also observed increased root yield due to complete defloration, but the alkaloid content per unit weight of dry root did not change with complete defloration.

Mass scale of collection of *R. serpentina* from natural habitats is leading to a depletion of plant resources. Therefore, efforts towards systematic cultivation, propagation, conservation and genetic upgrading for productivity and quality of existing genetic stocks are important (Mathur *et al.*, 1993). Barnah and Nath (2000) observed ecophysiological adaptability of *R. serpentina*.

Propagation of *R. serpentina* by means of seed is unsatisfactory, due to unfavourable climatic conditions. like varying temperature and humidity prevailing during the months of July and August which cause shrivelling and desiccation of the endosperm. This hinders the development of embryos and causes the formation of non-viable seeds of large number. The germination percentage of viable seeds is very low in the plant grown in plains of Darjeeling district. Thus there is enough scope to study germination behaviour of seed of the plant for their large scale propagation.

Other means of propagation are by root stumps and by root, stem and leaf cuttings. Of these the only method which gives a high percentage of successful plants is the propagation by root cuttings (Sitaram, 1978). However, the alkaloidal content of roots from plants raised by root cutting is less than those raised from seeds. Furthermore, the roots are not cherished for propagation by the growers due to their demand in the drug market

Biotechnology has slowly evolved during the early fifties and may be said to have come of age in agriculture only in the eighties. The pioneering researches include the discovery of phytochrome and its role in switching plant advanches permits splicing and annealing DNA sequences at all. Now a days the definitions are usually oriented towards processes and industry related products. Thus in 1981 European Federation of Biotechnology defined it as "integrated use of biochemistry, microbiology and chemical engineering in order to achieve the technological application in the capacities of microbes and cultured tissues. In 1982 organization of the Economic Corporation and Development defined biotechnology as the "application of scientific and engineering principle to the processing of materials by biological agents to provide good services".

Dr. T. B. Kenorcy, Jr. Administrator, Agricultural Research Service (USDA) (1986) defined it as "those biological means used to develop process and products employing organisms or their components" (Dasgupta, 1991).

The world population is nearing 5 billions and this rate of growth it is likely to touch 7.5 billions within a few years (Rajendra and D'Souza, 1999). The primary health care of the people is a necessity and the nature can only provide the needed resources. The *in vitro* culture of ayurvedic medicinal plants can be used for the supply of plants to the people and ayurvedic practitioners. The *in vitro* culture of plants with a view to getting secondary metabolites is not encouraging. Comparison made between whole plants and cell cultures show that using conventional whole plants is more practical and economical (Chem and Zu,

1996). Recently a large number of scientists are, therefore, engaged in micropropagation and *in vitro* preservation of plants known to have therapeutical properties.

Commercialization of tissue culture of medicinal plants, however, has received a poor response compared to that of ornamental plants. It is estimated that worldwide approximately only 5% of the total production of medicinal plants is through tissue culture. However, in India and other developing countries commercial production of medicinal plants through tissue culture is only 0.1% of the total production, though with lower wages plants are being produced at much cheaper rates. Though some medicinal plants are being produced commercially in India through tissue culture, but some important plants specially *Rauvolfia serpentina* are needed large quantities as they are in great demand (Rajendra and D'Souza, 1999), special attention and efforts have to be made for commercial exploitation of these plants.

There were previous attempts for the propagation of *R. serpentina* through tissue culture (Akram and Ilahi, 1986; Ilahi and Akram, 1987; Ilahi *et al.*, 1988; Akram *et al.*, 1993; Ruyter *et al.*, 1991; Roy *et al.*, 1995; Sarker *et al.*, 1996; Roja and Heble, 1996). The roots of the regenerated plants were compared with conventionally cultivated *Rauvolfia* plants for the presence of indole alkaloids (Ruyter, 1991). Vomelenine has been observed to be produced during cell culture of *R. serpentina* (Stockigt *et al.*, 1981; Chand *et al.*, 1999). Recently Akhtar *et al.* (2001) reviewed root culture of various medicinal plants with special emphasis on productivity of secondary metabolites though Benjamine *et al.* (1994) reported the production of reserpine during hairy root culture of *R. serpentina.* The hydroquinone; o-glucosyl transferase has been isolated from cultivated *Rauvolfia* cells (Arned *et al.*, 2000).

With this background investigation has been carried out on *R. serpentina* under various treatments and conditions in the ecological condition of Darjeeling district of West Bengal with the following objectives:

(i) Extraction and purification of reserpine available in *R. serpentina* and to develop an easy colorimetric method for its quantitative estimation.

(ii) To study the different methods of propagation of *R. serpentina.*

(iii) To study the growth and root and alkaloid yields of *R. serpentina* in relation to NPK fertilizers and irrigation.

(iv) To establish an efficient protocol for micropropagation of *R. serpentina* using different growth regulators. An attempt to isolate and estimate alkaloids from *in vitro* and *in vivo* grown plant materials was also undertaken.

ABSTRACT

The present investigation was carried with *Rauvolfia serpentina* which grows abundantly in the ecological conditions of Darjeeling District, West Bengal, India and yields highest amount of alkaloids as compared to other species of *Rauvolfia* available in India. It has been the objectives of the study to investigate some of the chemical and botanical aspects for its purposeful utilization in our society.

An important indole alkaloid reserpine was isolated, purified and characterised from root of *R. serpentina*. Dry powdered bark of *R. serpentina* was extracted with chloroform and was purified under column chromatography. The chemical was identified by paper chromatography and finally identification and characteization was done by IR spectrum.

For quantitative estimation of reserpine from root bark of *R. serpentina*, a new, easy and rapid colorimetric method was developed.

Owing to the hard sclerotic endocarp, the seeds of *R. serpentina* require longer time to germinate and the germination percentage is low. Effectiveness of different treatments for germination percentage of seeds of *R. serpentina* was studied.

Seeds scarification with sand paper was able to increase germination to some extent. But grinding of seeds with stone or nicking with a needle were not effective. The concentrated sulphuric and nitric acids and hot water treatment could not prove to be effective in increasing germination.

Pre-sowing seed treatment with chemicals did not improve germination. However, potassium nitrate and thiourea improved germination to some extent.

Although cultivation of *R. serpentina* through seeds is the most economic method, but it is extremely difficult to obtain the required supply of seed from natural sources. Moreover, germination percentage is very low and cannot be overcome by physical and chemical methods. So, there seems no other alternative than to undertake propagation by stumps, root and stem cuttings, which are more easily available.

The results of the present investigation indicated that stump cuttings had the highest root formation activity and it was followed by root and stem cuttings.2,4–D at very low concentration and IBA and NAA at low concentration stimulated root formation of stem cuttings. The highest activity (100%) was observed at 5 ppm of 2,4-D and at 100 ppm. it totally inhibited rooting activity of the stem cuttings.

The effect of time of planting and type of cutting on the success of cutting and subsequent growth of *R. serpentina* was studied. Both type of planting and type of cutting had significant effect on percentage of success, plant height, number of roots per plant, length of longest root and air-dried root weight. Planting on 15 June produced the highest percentage of success and plant height, maximum number of roots and higher root weight per plant. 15 March planting produced the lowest root yield. Root cuttings produced significantly higher root weight than the stem cuttings.

Under planting condition, regular supply of nutrients should be maintained for proper growth and higher yields of root and alkaloid of *R. serpentina*. Effect of different combinations and levels of NPK fertilizers on growth and yield of *R. serpentina* was studied. Five combinations of NPK fertilizers were T_0 ($N_0P_0K_0$), T_1 ($N_{30}P_{20}K_{15}$), T_2 ($N_{60}P_{40}K_{30}$), T_3 ($N_{90}P_{60}K_{45}$) and T_4 ($N_{120}P_{90}K_{60}$). Three levels of each of the fertilizers were N_{30}, N_{60}, N_{90}, P_{20}, P_{40}, P_{60}, K_{15}, K_{30} and K_{45}.

Leaf area per plant and total above-ground dry matter were increased with different combinations and levels of NPK fertilizers. Relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and relative leaf growth ratio (RLGR) responded much by the application of N, P and K, but on specific leaf area (SLA) and leaf weight ratio (LWR) were less pronounced. NAR had positive correlation with RGR and LAR. LAR had positive association with both its components, SLA and LWR. Root yield had positive correlation with RGR and NAR.

Chlorophyll content was increased by NPK fertilizers. Compared to P and K, the different levels of N had more effect on chlorophyll content. Root yield and alkaloid content were increased by NPK fertilizers.

Information is needed regarding the morphological and physiological responses of *R. serpentina* to water stress for cultivation as plantation crop. The effect of soil moisture on growth attributes, relative leaf water content, chlorophyll, proline and sugar contents and root and alkaloid yields was studied. Three levels of irrigation treatment were rainfed, irrigation one in every month and irrigation twice in every month.

Both leaf area and dry matter per plant were significantly increased with the increase of soil moisture. Compared to control, RGR of the irrigated plants were higher at the initial and the final harvest intervals, but lower at the middle harvest interval. Irrigation increased NAR. The plants under irrigated condition had higher RLGR than the rainfed ones. There were no clear pattern of soil moisture effect on SLA and LWR.

Relative leaf water content of the irrigated plants was significantly higher than the rainfed plants. Chlorophyll content was higher in the irrigated plants but proline and total free sugar was higher in the rainfed control.

Main root length, total alkaloid and reserpine contents were unaffected by soil moisture, but air-dried root yield increased with the increase of irrigation frequency.

As *R. serpentina* has poor seed viability and low germination percentage, tissue culture methods for propagation offer an effective and quicker way to overcome the obstacles for production of a large number of propagules. Tissue culture methods would be a valuable alternative for rapid propagation and conservation of this valuable threatened plant species.

In the present investigation, various *in vitro* aspects of tissue culture have been tried with different explants of *R. serpentina.* Young branches of field grown plants were used for primary establishment of cultures. For shoot proliferation, shoot tips and nodal explants from 8-9 months old field grown plants were used as explant sources.

It was observed that frequency of callus formation was greater in cultures of shoot tips than nodal explants. Formation of base callus significantly decreased the frequency of multiple shoot formation, number of shoots per explant and also shoot length.

By increasing the concentration of BA, the percentage of explant produced shoots increased and the optimum concentration was 2.0 mg/ l irrespective of concentrations of NAA used. GA_3 with BA and K_n did not increase the number of shoots per explant like BA with NAA and K_n with NAA. Optimal concentration of GA_3 was 0.1 mg/ l with 2.0 mg/ l BA and 0.5 mg/ l with K_n.

MS medium supplemented with different auxin-cytokinin combinations was used to study the callusing response. Field grown plants and *in vitro* grown plants were used for this purpose. It was observed that callus proliferation strictly depended on exogenous hormone supplementation. In absence of exogenous

hormone, explants failed to induce callus and became necrotic and died within a few days. Among the four auxins used, 2,4-D was found to be the best in respect of callusing response. On the other hand, BA was superior to K_n for callus growth when supplemented with 2,4-D.

The combination of 2,4-D and BA was the most effective formation for both explants (internode and leaves) used in the induction of callus. But IBA alone or either with BA or Kn was the least effective to induce callus formation.

Field grown explants failed to induce root formation when placed in a culture medium. However, shoot obtained from first and second subcultures failed to induce root formation. Rooting took place only when explants were taken from third sub-cultures. NAA was superior to other auxins when used singly or combinedly with other auxins for rooting. Further the percentage of cultures responded to rooting was always higher in shoot tip than nodal explants having one axiliary bud.

The results clearly show that both the shoot tips and nodal explants of *R. serpentina* plant are capable of producing multiple shoots *in vitro* and subsequently root to form complete plantlets.

In order to know and compare the content of reserpine in shoots and leaves of *in vitro* grown plants, reserpine content of field grown plants were also studied as a control. It was noted that *in vitro* grown plant parts possessed greater amount of reserpine compared to those in field grown plant parts. Almost 4 times reserpine were present in roots compared to shoot and leaves. Culture media also possessed some amount of reserpine.

CHAPTER 1

REVIEW OF LITERATURE

INFORMATION ABOUT NOMENCLATURE OF *Rauvolfia serpentina*

The dried root of *Rauvolfia serpentina* Benth. ex Kurz, commonly known as serpentina root, in Sanskrit as Sarpandha and in Hindi Chandrabhaga is one of the most important drugs used in modern medicine.

The genus *Rauwolfia* was named in honour of a sixteenth century traveller and botanist Leenard Rauwolf; serpentina refers to long tapering snake like roots. There have been various orthographies of the genus. It was Plumier who in 1703, named the genus *Rauvolfia.* However, Burmann in subsequent years revised the Plumier's work in 1760 and changed the spelling of the genus to *Rauwolfia* and that form prevailed untill very recent times. The original spelling *Rauvolfia* was ultimately restored following the provisions of Article 82 of the Botanical Nomenclature (1952) which conserves original spelling. (Wealth of India, 1969; Datta *et al.*, 1963).

MEDICINAL AND PHARMACEUTICAL USE OF *Rauvolfia serpentina*

R. serpentina is among the most important medicinal plants native to India. The roots of the plant have been used in the indigenous system of medicine from ancient times. The importance of the root drug and the alkaloids obtained from it has been recognized in the allopathic system in the treatment of hypertension or as a sedative and tranquillizing agent (Akram *et al.*, 1993; Roy *et al.*, 1996; Roja and Heble, 1996). A large number of alkaloids have been isolated

from the roots of this plant. The important among these are ajmalicine, ajmaline, ajmalinine, rescinamine, reserpine, reserpinine, serpentine, serpentinene and yohimbine. Detailed studies have been carried out on the chemistry of these alkaloids, their pharmacodynamics and their varying roles in essential hypertension and neuropsychiatric conditions. The findings especially those relating to the therapeutic action of reserpine attracted world wide attention and large quantities of roots were exported to USA and countries in Western Europe. As almost whole of the material comes from wild sources, the supplies declined sharply by 1952. In 1955, Government of India put a ban on the export of the raw drug and attempts to cultivate the plant were taken up at a number of places. A reassessment of the resources both from wild and cultivated sources has, therefore, been done and prospects for augmenting the supplies, have been examined in detail (Biswas, 1956; Chandra, 1956; Mukharjee, 1959, Rajgopalan, 1959). Rath *et al.* (1999) observed that the plant has got some effect similar to that of attenonol in allopathic medicine. According to Pravathi Devi (2000) the usage of the plant is safe without side effect like hormones and keeps the woman as feminine forever.

ECOLOGICAL ADAPTATION OF *R. serpentina* IN INDIA.

R. serpentina grows wild under a wide range of climatic conditions. It, however, prefers a tropical or sub-tropical belt having the benefit of monsoon rains averaging between 250 cm and 500 cm and the annual temperature ranging between 10°C and 38°C. The major soil types under the natural growth of the plant are sandy alluvial loam, red lateritic loam and in some places the stiff dark loam. A large percentage of humus, ensuring uniform moisture level is always associated with a good growth. The soils in most of the places are acidic having a pH ranging between 4 and 5.

In the sub-Himalayan zone, the areas lying to the west of Sirmor district are poor in the natural occurrence of the plant. In the central region, i.e., between Sirmor and Gorakhpur district of Uttar Pradesh, the plant is frequently noticed in shady moist or sometimes swampy localities. In the eastward localities in Bihar, north Bengal and Assam as well as in Khasi Jaintia and Garohills the plant is encountered more numerously on the forest margins of mixed deciduous forests. In the Western ghats, *R. serpentina* occurs more frequently in Goa, Coorg, North Canara and Shimoga Districts of Karnataka and Palghat, Calicut and Trichur Districts of Kerala. In Orissa, Andhra and Madhya Pradesh, the areas comprising the catchment of river Godavary are the richest. The plant is chiefly associated with Sal (*Shorea robusta*) forests as well as bamboo brakes (Raghavan Nair, 1955; Rajkhovas, 1967). Ecophysiological study of *R. serpentina* has been done by Barnah and Nath (2000).

TRADE AND COMMERCE OF *R. serpentina* IN INDIA

The major trade centres dealing with *Rauvolfia* roots are Kolkata, Mumbai and Patna which in turn are fed by a number of primary trade centres, viz. Dehra Dun, Ramnagar and Tanakpur in U.P., Hazari Bagh and Patna in Bihar, Coochbehar in West Bengal, Gauhati in Assam, Tura in Meghalaya, Bhavanipatna in Orissa, Vishakhapatnam in Andhra, Sirsi in Maharashtra and Palghat in Kerala where material from a particular growing area are received. The drug in commerce is sold under various trade names, viz., Assam, Malabar, Canara, Orissa, Bihar, Himalayan and Dehra Dun varieties. The materials from Bihar, Canara and Dehra Dun fetch a higher price (Sulochna, 1959; Vardvajaw, 1963).

CHEMISTRY OF *Rauvolfia* ALKALOIDS

The number and quantity of alkaloids isolated from *R. serpentina* and numerous other species vary widely even in the same species. More than 20 indole

alkaloids have been isolated so far from *R. seprentina* and most of the alkaloids are confined to the roots of the plant. The total alkaloid contents in the root range from 1.7 to 3.0% which are mostly concentrated in the bark (about 90%). Alkaloids are also present in leaves, stems and seeds, but not in significant amount as compared to roots.

The first report concerning hypotensive properties of *Rauvolfia* extracts was made by Chopra *et al.* in 1933. This observation together with the increasing use of the drug as a sedative, stimulated much interest in solving alkaloid from *Rauvolfia*. Although several bases were isolated and investigated, none of them appeared to possess the therapeutic value of the crude extracts. It was later shown that the sedative principle of *R. seprentina* resided in the 'oleoresin' fraction and was presumed to be due to a non-alkaloidal component (Dutta *et al.*, 1947). Investigation on this oleoresin fraction was initiated by Muller and coworkers, who succeeded in isolating the indole alkaloid reserpine (Muller *et al.*, 1952), which was shown to be the principal hypotensive and sedative ingredient of *R. serpentina* (Akram *et al.*, 1993; Roy *et al.*, 1995; Roja and Heble, 1996).

SPECIALITY OF CHEMICAL STRUCTURE OF INDOLE ALKALOIDS

With a few minor exceptions, tryptophan and its decarboxylation product, tryptomine, give rise to the large class of indole alkaloids. These bases usually contain two nitrogen atoms of which one is indolic in nature. Of the several alkaloid groups within the indole class, two may be produced depending on the type of concentration occurring between tryptomine and an aldehyde or ketoacid. A Mannich reaction involving the alpha carbon atom of the indole nucleus affords a beta-carboline derivative; reaction involving the beta position gives rise to an indolenine.

Thus, out of the two nitrogen atoms in the indole alkaloids, one is secondary in nature (R_1NH) and the other is in the form of tertiary amine (R_2N). Since the nitrogen atom bears an unshared pair of electrons, such compounds are basic and resemble ammonia in chemical property. The degree of basicity varies greatly depending upon the structure of the molecule and presence and location of other functional groups. Like ammonia, the alkaloids are converted into their salts by aqueous mineral acids and when the salt of an alkaloid is treated with hydroxide ion, nitrogen gives up a hydrogen ion and the free amine is liberated. The positive charge of the nitrogen ion depends on the presence of how many organic groups are covalently bonded to nitrogen and positive charge of this ion is balanced by some negative ion [$R_3N^+X^-$]. If the nature of ammonium ion is such that there is no proton to give up, it will not be affected by hydroxide ion. Consequently, the compounds will have chemical properties quite different from those of the amines. For the most part of the alkaloids are insoluble or sparingly so in water but the salts formed upon reacting with acids are usually freely soluble. The free alkaloids are usually soluble in ether or chloroform or other relatively nonpolar, immisible solvents in which, however, the alkaloidal salts are insoluble. This permits a ready means for the isolation and purification of indole alkaloids.

ALKALOIDS ISOLATED FROM DIFFERENT SPECIES OF *Rauvolfia* WITH SPECIAL INTEREST ON *R. serpentina*

Siddiqui and Siddiqui (1931) were able to isolate five different types of alkaloids from the root of *Rauvolfia serpentina* Benth. They were able to determine their molecular formulae and few other characteristics as (i) ajmaline ($C_{20}H_{26}O_2N_2$. $3H_2O$). m.p.159°–160°, (ii) ajmalinine ($C_{20}H_{26}O_3N_2$. $1.5H_2O$). m.p.180°–181°, (iii) ajmalicine m.p.250°–252°, (iv) serpentine ($C_{20}H_{20}O_3N_2$. $1.5H_2O$). m.p.263°–264° and (v) serpentinine ($C_{20}H_{20}O_5N_2$. $1.5H_2O$). m.p.263°–265°C and their contents in the plant root were 0.1%, 0.25%, 0.02%, 0.08% and 0.8% respectively.

Siddiqui and Siddiqui (1935) studied the ajmaline series of alkaloids of *R. serpentina*. They showed that ajmaline takes up 2 atoms of bromine in the cold yielding a crystalline dibromo derivative, which indicates the presence of an olefine double bond in its molecule. It also forms a sulphonic acid, characterized through its salts, and a tri-nitro derivative. From the crystalline product obtained on heating ajmaline to 200°C and provisionally named in its crude and undefined condition as pyroajmaline, it was possible to isolate after repeated crystallisation from ethyl acetate some unchanged ajmaline (m.p. 158–160°C) but mainly a product which melts at 265–266°C, agreeing with ajmaline in its analysis, colour reactions and NH and N-Me groups.

Siddiqui (1939), working on the roots and root bark of *R. serpentina* collected from the more temperate climate of the Dun Valley, reported the presence of two isomers of ajmaline, namely isoajmaline and neoajmaline.

Schlitter and Schwarz (1950) however, were able to isolate only two alkaloids viz., ajmaline and serpentine from the roots of *Rauwolfia serpentina* Benth.

Bose (1956) isolated a new alkaloid, serpinine, $C_{20}H_{24}ON_2$ from *R. serpentina*. Its colour reactions, properties, UV and IR spectra were examined. He carried out several degradative experiments. From the available experimental data, a hexahydro-*ß*-Carboline structure of serpentine was suggested. It was found to be closely related to ajmaline in properties and constitution. The U.V. curve of serpinine, which was studied in ethanol, resembled very closely those of the indoline. It showed λ_{max} at 250 mμ and 293mμ and λ_{min} at 227 and 272mμ respectively. The infrared (IR) spectrum of serpinine exhibited absorption bands at 6.25μ (indoline nucleus), 6.84μ (phenyl nucleus), 7.24μ (C-Me), 7.4μ (N-Me) and 13.55μ (o-distributed phenyl).

Bose *et al.* (1956) studied the alkaloid contents of *R. beddomei*, a plant indigenous to South India. They isolated sarpagine from the roots of *R. beddomei*.

Alkaloids were drained from the powdered roots by cold percolation with ethyl alcohol. Chromatographic resolution of a benzine solution of this crude base over Merck's alumina furnished an alkaloid, $C_{22}H_{24}O_3N_2$, m.p.257°C. From the infra-red (peak absorption at 5.87 and 6.16μ) and ultraviolate data (increased absorption at 250mμ) of this alkaloid, the existence of a *ß*-alkoxy-cyclic ester group has been concluded. The infra-red spectrum also exhibited bands at 2.85μ (>NH), 7.25μ (C–CH_2) and 9.05μ (ether bridge). The alkaloid was shown to be a tetrahydro-*ß*-carboline derivative from evidence of colour reactions.

Datta (1956) in a pharmacognostic investigation on the following species - *Rauwolfia serpentina, R. canescens, R. heterophylla, R. hirsuta, R. densiflora, R. decurva and R. perakensis* found positive test for the presence of reserpine in the extract of root powder in all the species except *R. densiflora, R. decurva* and *R. perakensis.*

Siddiqui (1958) studied the alkaloids of *R. serpentina* and the mode of their occurrence. The procedure adopted for the isolation of therapeutically active constituents in their naturally occurring complex form offered a new approach to studies in medicinal plant materials. The substances isolated were petroleum ether soluble oleo-alkaloid fraction—resajmaline—greenish, viscous oily liquid containing fatty matter serposterol, and unsaturated higher alcohols along with around 2.3% reserpine and 0.5% rescinnamine and traces of ajmaline. Ethyl acetate–benzene soluble alkaloidal complex–ajmalexine–forming a cream coloured powder with a concentration of the weaker *Rauwolfia* bases including 5.5% of rescinnamine and some unknown substances. Serpajmaline fraction, soluble in water, mainly contained the stronger bases serpentine, serpentinine, ajmaline and two unknown substances.

Siddiqui *et al.* (1959) studied the action of bromine on ajmaline and its various derivatives to elucidate the mechanism of this reaction and earlier points of disagreement in respect of the chemical characteristics of ajmaline had been experimentally checked up for clarification. Further, on the basis of studies in the

action of cyanogen bromide on the diacetyl derivatives of ajmaline and hexahydroajmaline, the antifibrillant cardiac action of ajmaline was correlated with the N-stability of the carbinolamine structure, which appeared to function as a cardiophore grouping in the ajmaline molecule.

Timmins and Court (1976) isolated indole alkaloids alstonine, 10-methoxygeissoschizol, tetrahydroalstonine, vomalidine, α-yohimbine and 19, 20–dehydroyohimbine, an unidentified anhydronium-like base and choline from *R. obscura* stems. The diester alkaloids reserpine and rescinnamine, which occur in roots, were not detected.

Iwu and Court (1978a) reported that the leaves of *R. cumminsii* yielded at least 12 indole alkaloids. Two E-seco indole alkaloids (corynantheol and corynantheal), 7 ajmalan type dihydroindole alkaloids (endolobine, norpurpeline, dihydronorpurpeline, normitordine, norseredamine, nortetraphyllicine and seredamine–17–0–(3', 4', 5') trimethony benzoate), 2 sarpagan alkaloids (normacusine β–0–methyl and an incompletely characterized compound) and the indoline alkaloid picrinine were isolated. The apparent scarcity of Na^- methylated indoline alkaloids in the plant may prove to be significant.

Iwu and Court (1978b) isolated 18 indole alkaloids from *R. cumminsii* roots and 17 were characterized. The alkaloids comprised sarpagan, yohimbine, 18-hydroxy-yohimbine, heteroyohimbine, anhydronium, α-acyl indole and dihydroindole types. Dihydroindoles were not previously reported in *R. cumminsii* roots.

Iwu and Court (1978c) isolated 24 indole alkaloids from the stem bark of *Rauwolfia comminsii* and among these 21 were identified. The alkaloids comprised E-seco, sarpagan, dihydroindole, yohimbine, heteroyohimbine, 18-hydroxyyohimbine ester and anhydronium types together with peraksine and deacetylpicraline. The probable biosynthesis of alkaloids was discussed.

Sabri and Court (1978) isolated 22 indole alkaloids from the stem bark of Nigerian *R. vomitoria* and 20 of them were characterized. The alkaloids comprised E-seco heteroyohimbine, sarpagan, dihydroindole, yohimbine and heteroyohimbine types.

Amer and Court (1980) isolated nineteen alkaloids from Ghanaian *R. vomitoria* leaves. The alkaloids comprised E-seco indole, sarpagan, picrinine, akuammiline, heteroyohimbine, oxindole, yohimbine and indolenine types.

Akinloye and Court (1980a) isolated and identified twenty-one indole alkaloids from the leaves of *R. oreogiton*. The alkaloids comprised E-seco heteroyohimbine, heteroyohimbine, akuammiline, akuammicine, pleiocarpamine, picraline, picrinine, dihydroindoline and sarpagan types. No chemical differentiation between the leaves of *R. oreogiton* and *R. volkensii* could be established.

Akinloye and Court (1980b) isolated and identified thirteen alkaloids from the leaves of *R. volkensii*. The alkaloids included E-seco heteroyohimbine, heteroyohimbine, sarpagan, dihydroindoline, pleiocarpamine, picrinine and akuammicine types together with peraksine.

Lastra *et al.* (1982) isolated three alkaloids, viz., aricine, tetrahydroalstonine and vellosinine from the leaves of *R. cubana*. These alkaloids were also identified. No reserpine was detected in the roots of *R. tetraphylla*, whereas ajmaline was found in the roots. Reserpine (0.04%) and ajmaline (0.07%) were isolated from the roots of *R. cubana*. Roots of *R. cubana* showed pharmacological activity attributed to Rauvolfia alkaloids.

Nasser and Court (1983) isolated eighteen alkaloids from South African *Rauwolfia caffra* and these were corynane, sarpagan, peraksine, akuammicine, macroline, indolenine and harman types. Heteroyohimbines and dihydroindoles were not detected. The principal alkaloids were the indolenine compounds

raucaffrinoline, perakine and vomilenine and the indole alkaloids peraksine and dihydroperaksine.

Court (1983) reported the distribution of indole alkaloids in the leaves, stem and roots of 10 African mainland *Rauwolfia* species such as *R. oreogiton, R. volkensii, R. affroa, R. macrophylla, R. mannii, R. obscura, R. rosea, R. cumminsii, R. mombasiana and R. vomitoria.* He also discussed the interrelationships of the alkaloid types.

Nasser and Court (1983) reported the alkaloids *R. caffra* seeds. The results indicated that seeds yielded 0.012% total alkaloids comprising the sarpagan compound normacusine β, α-yohimbine, alloyohimbine, an incompletely characterized yohimbine. Chromatographic evidence indicated traces of nonajmalan and ajmalan compounds.

Siddique *et al.* (1985) isolated a new dihydroindole alkaloid sandwicoline from undried winter roots of *R. serpentina* of Nepalese origin. Its structure was determined as 21-monohydro-N-methyl-sandwicine through chemical and spectral studies.

Kan *et al.* (1986) isolated four monomeric indole alkaloids from the bark of *Rauvolfia media.* Three of them were the known cabucine, reserpiline and mauiencine and the fourth was a new alkaloid 12-hydroxymauiensine.

Siddique *et al.* (1986) determined the structure of a new yohambanoide, rescinnaminol ($C_{32}H_{42}N_2O_6$ m.p. 241–43°C). This alkaloid was isolated from *R. serpentina* roots and was elucidated through spectroseopic methods.

Siddique *et al.* (1987a) reported that the alcoholic extract of the roots of *R. serpentina* had been elucidated as cyclonexy ester of indolepropionic acid by spectral and chemical studies.

Siddique *et al.* (1987b) enlisted two hundred and four alkaloids isolated from various *Rauwolfia* species and their molecular formulae, melting points and specific rotation were presented.

Siddique *et al.* (1987c) isolated a new alkaloid yohambinine from the alcoholic extract of *R. serpentina* roots and was identified as 5 beta–methylpseudoyhimbane by spectroscopic studies. It is the first 5–methyl yohimbanoid isolated from any plant.

Siddique *et al.* (1987d) elucidated the structure of a new base, rescinnamidine ($C_{35}H_{44}N_2O_9$, m.p. 260–61°C) isolated from the roots of *R. serpentina* as 2', 3'–dihydrorescinnamidine.

Siddique *et al.* (1987e) isolated a new dihydroindole alkaloid ajmalinimine from the roots of *R. serpentina* collected from Thailand. Its structure was determined as 10–C, 17–O–diacetylajmaline on the basis of chemical and spectroscopic studies.

Siddique *et al.* (1987f) were able to identify a new heteroyohimban alkaloid-ajmalicidine, from the roots of *Rauwolfia serpentina* of Thi-origin. Its structure was elucidated as 1–carbomethoxy–17 α–hydroxy–16–decarbomethoxy 16, 17–dihydro-ajmalicine through chemical and spectral studies.

Intipoua *et al.* (1988) studied alkaloid composition in the roots, stem and leaves of *Rauwolfia caffra, R. cansescens, R. heterophylla, R. serpentina* and *R. verticilata.* Ajmaline alkaloids were accummulated in the roots but reserpine alkaloids were found in all parts of the plants. Recommendations were made for using *R. cansescens* for commercial growing as a species with the highest alkaloid content.

Schuibel *et al.* (1989) observed the production of gluco-alkaloid raucaffricine in cell suspension culture of *Rauwolfia serpentina.* The alkaloid production medium was modified by adding 100 g sucrose and 2.5 g

$MgSO_4.7H_2O$/1 of the medium. The culture produced after 18 days upto 1.6 g raucaffricine when the medium was inoculated with 200 g of cell grown for 10 days in Linsmaier–Skoog medium. This yield exceeded that was known from intact plants (*Rauwolfia caffra*) by factor of 12.

Nikolaeva *et al.* (1990) presented data on ajmaline content in various organs of the following *Rauwolfia* spp. Grown in the Transcaucasus Georgian SSR, USSR: *R. caffra, R. canescens, R. heterophylla* and *R. serpentina*. Data were also given on ajmaline content in the root bark of the following species from the flora of Vietnam : *R. cambodiana, R. cansescens, R. serpentina, R. verticillata, R. vomitoria and R. littoralis, R. vomitoria* and , *R. cansescens* were the most valuable species with respect to ajmaline content.

Falkenhagen *et al.* (1992) cultured the hairy roots of *R. vomitoria* in hormone-free B5 medium. The six weeks old cultures were used for phytochemical analysis. The major alkaloids detected were vinerine, perakine, ajmaline, asmalinol and a yohimbine-isomer. Structures of all the alkaloids were detected, except ajmalinol, which was reexamined.

Endresse *et al.* (1992) observed that when ajmaline, one of the major alkaloids of *R. serpentina* was added to *R. serpentina* cell suspension culture, a new series of alkaloids was formed. Five novel alkaloids were isolated from these ajmaline-fed cell cultures and their structures were deduced. Four of the novel products belonged to the raumacline group: 6α–hydroxy aumacline, 6α–methoxy-raumacline, 19–hydroxy–N–methylraumacline and 20–isoraumacline. The structure of a further new alkaloid was different from the raumaclines and belonged to the sarpagine alkaloids.

Endresse *et al.* (1993) isolated a group of new alkaloids, the raumaclines, and some related alkaloids from *R. serpentina* cell suspensions fed with high levels of ajmaline; their structures were determined and sysnthesis developed

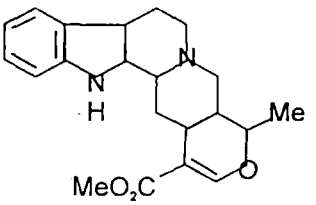
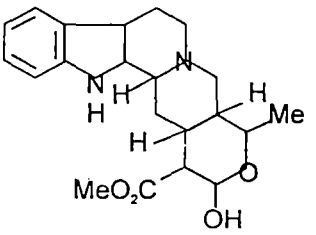
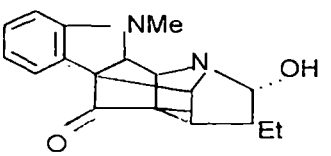
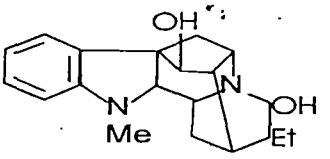
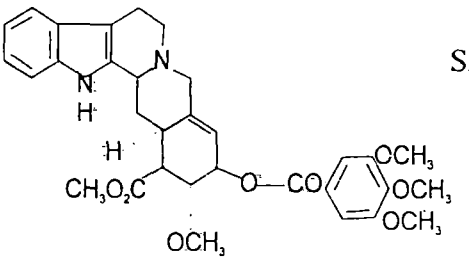
providing an essential prerequisite to the further study of their biosynthesis at the enzymatic level.

Bianco *et al.* (1994) isolated from the bark of *Rauwolfia grandiflora* a new monoterpenoid δ–lactone, isoboonein, together with boonein, loganin and loganic acid. The structure of isoboonein, established by spectroscopical methods, was confirmed by partial synthesis from loganic acid.

Ferreira Batista *et al.* (1996) obtained a new alkaloid, sellowiine (N-demethyl-20-deethyl suaveoline), from leaves of *R. sellowii*, collected at two different locations in Southern Brazil. They also obtained the known alkaloids, perakine, raucffrinoline, vomilenine, 19α, 20α–epoxy–akuammicine, picrinine and 12–demethoxytabernulisine. The NMR spectra of the alkaloids were assigned completely.

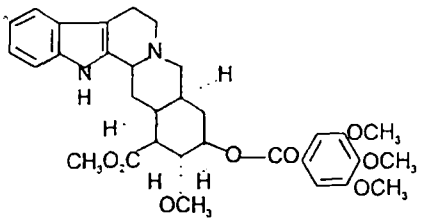
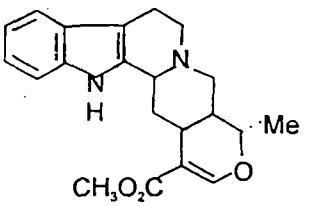
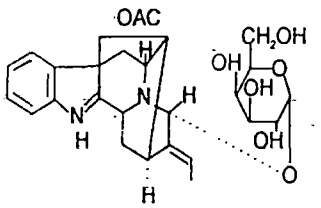
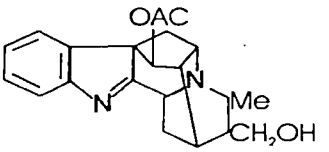
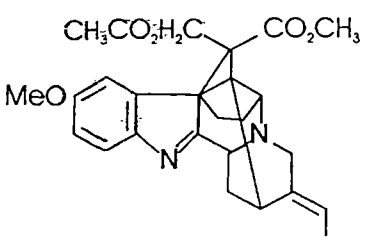
Some important alkaloids in different species of *Rauvolfia* including *R. serpentina* have been presented in Table 1.

Table 1 : Some important alkaloids isolated from *Rauvolfia* with special interest on *R. serpentina*.

Name of alkaloid, Name of the plant' Empirical formula & Melting point (°C)	Chemical structure	Reference
1 Ajmalicine *R. serpentina* $C_{21}H_{24}N_2O_3$ 253-4		Janot & Le Men, 1956 and Sharma & Moss, 1961
2 Ajmalicinine *R. serpentina* $C_{21}H_{26}N_2O_4$ 245-6		Bombardelli *et al.*,1974
3 Ajmalidine *R. sellowii* $C_{20}H_{24}N_2O_2$ 241-2		Prakash *et al.*,1955; Bartlett *et al.*, 1962
4 Ajmaline *R. serpentina* *R. vomitoria* $C_{20}H_{26}O_2N_2$ 205-7		Siddiqui and Siddiqui, 1931; Mukherji *et al.*, 1949; Chatterjee & Bose, 1954
5 Ajmalinine *R. serpentina* *R. vomitoria* $C_{20}H_{26}O_3N_2$ 180-1		Siddiqui and Siddiqui, 1931; 1932, 1935
6 Deserpideine *R. nitida* $C_{32}H_{36}O_8N_2$ 149-52		Smith *et al.*, 1964, 1967

Contd.

Table 1 (Contd)

7	Deserpidine *R. canescence* $C_{32}H_{38}O_8N_2$ 228-32		MacPhillamy *et al*, 1953; Neuss *et al.*, 1955
8	Raubasine *R. serpentina* $C_{21}H_{24}O_3N_2$ 256-7		Klohs *et al.*, 1954; Shamma and Richey, 1963; Finch *et al.*, 1966
9	Raucaffricine *R. caffra* $C_{27}H_{32}O_8N_2$ 220		Khan *et al.*, 1965
10	Raucaffridine *R. caffra* $C_{21}H_{24}O_3N_2$ 221		Khan *et al.*, 1965
11	Raucaffriline *R. caffra* $C_{21}H_{22}O_3N_2$ 200-01		Khan *et al.*, 1965
12	Raucaffrinoline *R. caffra* $C_{21}H_{24}O_3N_2$ 236		Khan and Siddiqui, 1972
13	Raufloricine *R. confertiflora* $C_{24}H_{28}O_5N_2$ 190-2		Danieli *et al.*, 1972

Contd.

Table 1 (Contd)

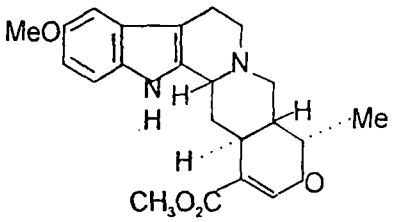
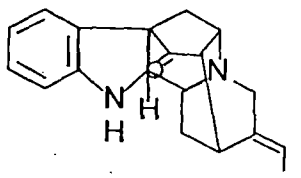
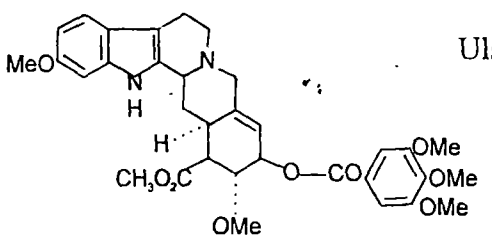
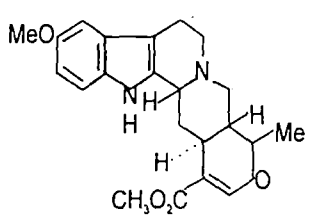
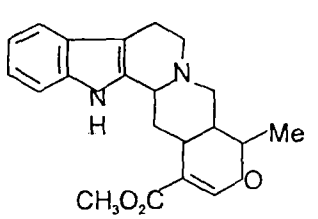
14	Raufloridine *R. confertiflora* $C_{22}H_{26}O_4N_2$ Indefinite		Danieli *et al.*, 1971
15	Rauflorine *R. confertiflora* $C_{19}H_{20}ON_2$ 221		Danieli *et al.*, 1971
16	Raugalline *R. serpentina* $C_{21}H_{28}O_3N_2$ 185		Le Gall, 1960
17	Rauhimbine *R. serpentina* $C_{21}H_{26}O_3N_2$ 218-25		Hoffmann and Helv, 1954
18	Raujemidine *R. canescens* $C_{33}H_{38}O_9N_2$ 144-5		Ulshafer *et al.*, 1956
19	Raumitorine *R. vomitora* $C_{22}H_{26}O_4N_2$ 138		Poisson *et al.*, 1954; Shamma and Richey, 1963; Finch *et al.*, 1966
20	Rauniticine *R. nitida* $C_{21}H_{24}O_3N_2$ 233-5		Salkin *et al.*, 1961; Shamma and Richey, 1963; Finch *et al.*, 1966

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Table 1 (Contd)

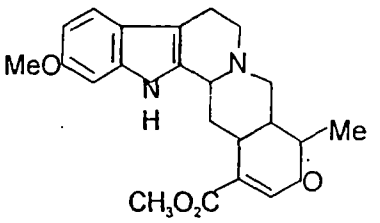
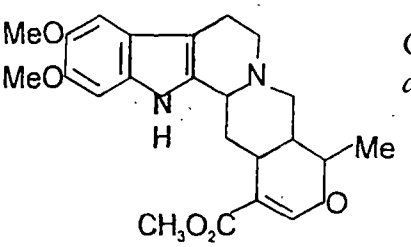
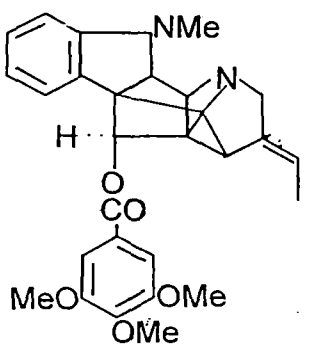
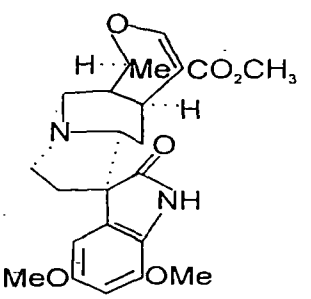
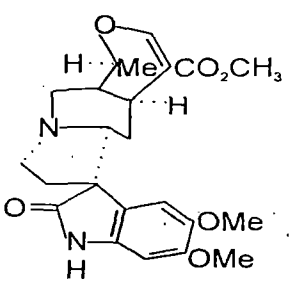
21 Raunitidine *R. serpentina* $C_{22}H_{26}O_4N_2$ 276-8		Salkin *et al.*, 1961; Shamma and Richey, 1963
22 Raupine *R. serpentina* $C_{20}H_{26}O_3N_2$ 325		Bodendorf and Eder, 1953
23 Rauvanine *R. vomitora* $C_{23}H_{28}O_5N_2$ 135		Goutarel *et al.*, 1961; Finch *et al.*, 1966
24 Rauvomitine *R. vomitora* $C_{30}H_{34}O_6N_2$ 115-7		Haack *et al.*, 1955; Poisson *et al.*, 1955; Bartlett *et al.*, 1962
25 Rauvoxine *R. vomitora* $C_{23}H_{28}O_6N_2$ 210-1		Patel *et al.*, 1964; Pousset and Poisson, 1964
26 Rauvoxinine *R. vomitora* $C_{23}H_{28}O_6N_2$ 203		Patel *et al.*, 1964; Pousset and Poisson, 1964

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Table 1 (Contd)

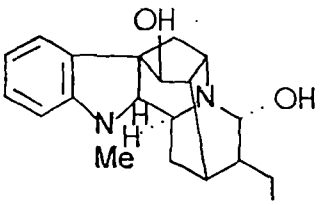
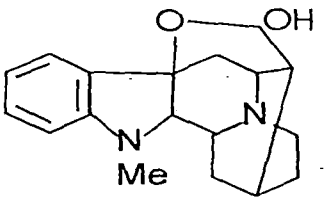
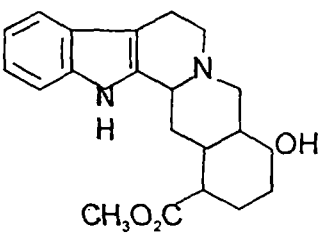
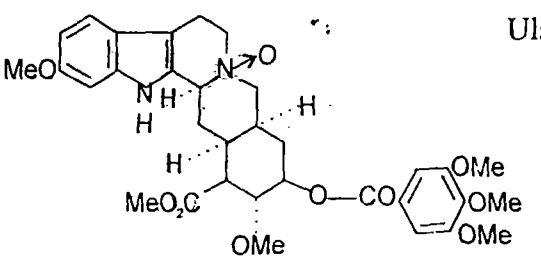
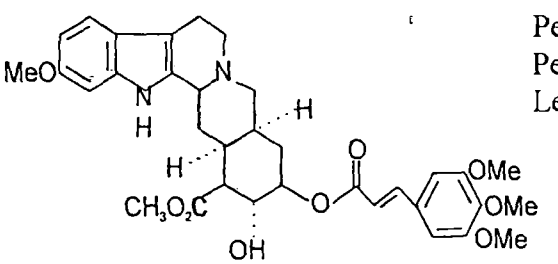
27 Rauwolfine *R. serpentina* *R. caffra* $C_{20}H_{26}O_2N_2$ 235-8		Itallie and Steenhauer, 1932; Koepfli, 1932.
28 Rauwolfinine *R. serpentina* $C_{19}H_{26}O_2N_2$ 231-3		Bose, 1954
29 Rauwolscine *R. canescens* $C_{21}H_{26}O_3N_2$ 231-2		Mukherjee, 1941, 1946
30 Renoxydine *R. vomitora* $C_{33}H_{40}O_{10}N_2$ 238-41		Ulshafer *et al.*, 1957
31 Rescidine *R. vomitora* $C_{34}H_{40}O_9N_2$ 183-6		Peopelak *et al.*, 1961; Peopelak and Lettenbauer, 1963

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Table 1 (Contd)

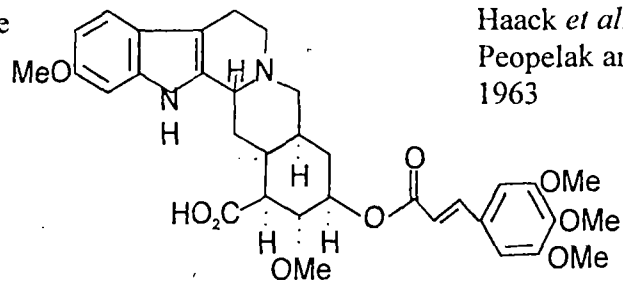
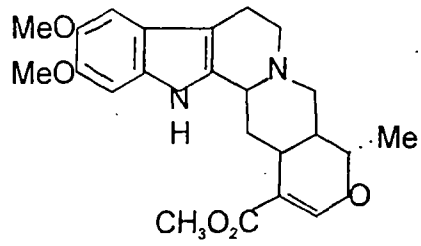
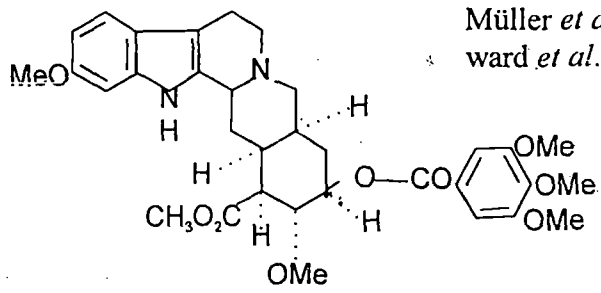
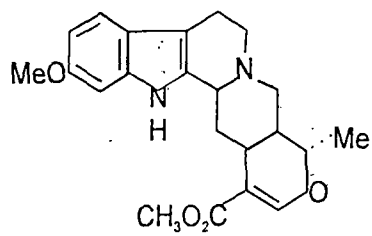
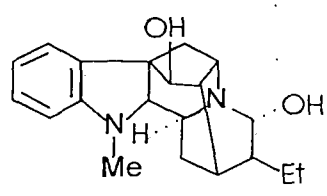
32	Rescinnamine *R. serpentina* *R. vomitora* $C_{34}H_{42}O_9N_2$ 238-9		Haack *et al.*, 1954; Peopelak and Lettenbauer, 1963
33	Reserpiline *R. serpentina* *R. canescens* $C_{23}H_{28}O_5N_2$ Indefinite		Klohs *et al.*, 1954; Stoll *et al.*, 1955; Shamma and Richey, 1963
34	Reserpine *R. serpentina* $C_{33}H_{40}O_9N_2$ 262-63		Müller *et al.*, 1952; Woodward *et al.*, 1958
35	Reserpinine *R. serpentina* $C_{22}H_{26}O_4N_2$ 243-4		Janot and Le Men, 1954; Schlittler *et al.*, 1954; Shamma and Richey, 1963.
36	Sandwicensine *R. sandwicensis* $C_{19}H_{22}ON_2$ 260-2		Gorman *et al.*, 1957
37	Sandwicine *R. sandwicensis* *R. mauiensis* $C_{20}H_{26}O_2N_2$ 220-2		Gorman *et al.*, 1957; Ronchetti *et al.*, 1971

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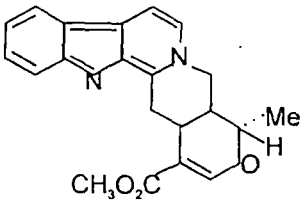
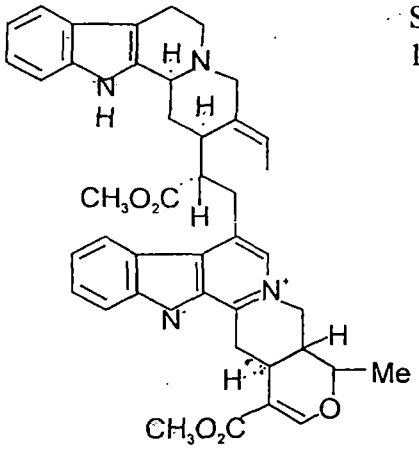
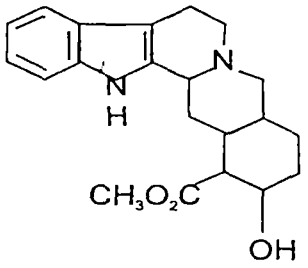
Table 1 (Contd)

38	Seredine *R. vomitora* $C_{23}H_{30}O_5N$ 291		Poisson *et al.*, 1954; Goutarel *et al.*, 1954
39	Serpentine *R. serpentina* $C_{21}H_{20}O_3N_2$ 158		Siddiqui and Siddiqui, 1931, 32, 35
40	Serpentinine *R. serpentina* $C_{42}H_{44}O_5N_4$ 263-5		Siddiqui and Siddiqui, 1931, 32, 35
41	Serpine *R. serpentina* $C_{21}H_{26}O_3N_2$ 215		Chatterjee and Bose, 1954
42	Serpinine *R. serpentina* $C_{20}H_{24}ON_2$ 315-7		Bose, 1954, 1955
43	Serpagine *R. beddomei* $C_{22}H_{24}O_3N_2$ 257		Bose, et al., 1956

METHODOLOGY FOR QUANTITATIVE ESTIMATION OF INDOLE ALKALOIDS IN *Rauvolfia* SPECIES

Shah and Hossain (1965) described a spectrophotometric method for the determination of the amount of serpentine in serpajmaline. The absorbance of serpentine in 5N acetic acid at 307mμ was used for the submicro spectrophotometric determination of this substance in serpajmaline which is predominantly a mixture of serpentine, serpentinine and ajmaline. Serpentinine which also absorbed at 307mμ was separated from the complex through electrophoresis. The extent of interference due to ajmaline in the UV absorbance measurements of serpentine was determined. The method was accurate within ± 1–2%.

Cieri (1983) presented for the identification estimation of some of the alkaloids of *R. serpentine* by high performance liquid chromatography (HPLC) and TLC. Rescinnamine was detected at 330 nm, at which wavelength reserpine fluorescence was negligible. Reserpine was detected at 280 nm, where rescinnamine fluorescence was small. Other alkaloids detected were raubasinine, ajmalicine, yohimbine, ajmaline and serpentine. For TLC, $CHCl_3$–CH_3OH (97+3) and $CHCl_3$–CH_3OH (80+20) were used as developing solvents and spots were detected under long and short wave UV light. A semiquantitative TLC procedure was also developed for serpentine, the content of which was in the 0.2–0.25% range.

Shimolina *et al.* (1984a) reported the quantitative determination of total alkaloids in *R. serpentina* tissue culture. Comparative data were presented on the weight, volumetric and extraction–photometric methods in the determination of total alkaloids. The volumetric method was recommended as the most objective and the least time–consuming method.

Shimolina *et al.* (1984b) developed a method for the determination of vomilenine and a closely related alkaloid on thin layer of silica gel with subsequent determination by elution spectrophotometric method. Maximum vomilenine content observed was 0.50–0.65%.

Nguyen *et al.* (1989) developed spectrophotometric and extraction photometric methods for determining ajmaline, reserpine and serpentine in the root bark of *R. serpentina, R. littoralia, R. cambodiana, R. verticillata, R. vomitoria and R. canescens*. The methods were simple, accurate and reproducible. High contents of the alkaloids was established in all samples. Recommendations were made for using *Rauwolfia spp.* growing in Vietnam as sources of raw materials in reserpine and ajmaline production.

Gubar *et al.* (1993) developed a rapid method for estimation of submicroquantities of alkaloids in cultured cells and studied alkaloid accumulation in *Rauwolfia serpentina* tissue culture. Quantitative method for estimation of indole alkaloids was elaborated by microcolumn chromatograph. The developed method permitted carrying out simultaneous determination of ajmalicine, reserpine, vomilenine, ajmaline and serpentine in the tissue culture extracts. Various *Rauwolfia* cell lines were analysed. About 30 different alkaloids were detected and contents of some alkaloids were measured in the cultured cells including alkaloids used in medicine, such as ajmalicine, reserpine, ajmaline and serpentine.

Cieri *et al.* (1987) established a method for the determination of reserpine and rescinnamine in *R. serpentina* powder or tablets by liquid chromatography (LC) with fluorescence detection. The sample was dispersed in CH_3OH, 0.5N and H_2SO_4 was added and the mixture was extracted with chloroform. The extracts were separated from interfering materials on a celite–0.1N NaOH column and the elutes were collected in 50 ml CH_3OH. After complete removal of the $CHCl_3$, reserpine and rescinnamine were determined by liquid chromatography on a normal phase column with CH_3OH as mobile phase.

A simple sensitive and highly specific method involving fluorescence analysis of reserpiline in highly acidic solutions was described by Balon-Almeda *et al.* (1986). The method could be employed for determination of reserpiline in commercial reserpine-rescinnamine preparations.

A radioimmuno assay (RIA) was developed for the individual measurement of serpentine in plant extracts. Each RIA was assessed for its sensitivity, specificity and accuracy. The method involving preparation of antigens and antibodies specific for these alkaloids as well as the radiochemical preparation of serpentine was successfully applied in the selection of individual plants with higher than average contents of these alkaloids for breeding and tissue culture purposes (Arens *et al.*, 1978).

BIOSYNTHETIC PROCESSES OF INDOLE ALKALOIDS

Any approach to improve indole alkaloid production requires through knowledge of the alkaloid biosynthetic pathway, enzymology and genetic regulation. The biosynthesis of indole alkaloids has been intensively studied especially during the last four decades. Although progress has been achieved in understanding the chemical make up of different indole alkaloids, but the accumulation of knowledge in connection with biosynthesis of these types of alkaloids not satisfactory, though certain main intermediates are well known. As there are certain problems in understanding the process of biosynthesis and their relationship between various groups of alkaloids, further work in this line is very much needed as complete picture is still not available.

Most of the work has been done with *Catharanthus roseus*, because the species has many advantages for biosynthetic studies. The incorporation of tryptophan into all major classes of indole alkaloids was shown by Leete's group (Leete, 1961; Yamazaki and Leete, 1965). Following tracer technology with the help of [2-C^{14}]–tryptophan specially lebelled reserpine and serpentine in *R.*

serpenitina and vindoline in *Catharanthus roseus* could be possible. The label form [3-C^{14}]–tryptophan was located at the predicted site in ibagaine (*Tabernanthe ibago*). But to find out the pathway, which is operative in nature apparently, require studies at the enzymatic level.

SOME RECENT OBSERVATIONS ON ENZYME ACTIVITY IN BIOSYNTHETIC PROCESS OF INDOLE ALKALOIDS AND THE ROLE OF PLANT TISSUE CULTURE

It is now possible to understand that vast majority of indole alkaloids are biosyntherised in plants follow shikimate and mevalonate pathways (Fig-1). Indole alkaloids consist of tryptamine provided by tryptophan (from shikimate pathway) and a terpenoid part provided by the iridoid glucoside secologanin (from mevalonic acid pathway). The first enzyme involved in undole alkaloid biosynthesis is tryptophan decarboxylase (TDC), which converts amino acid tryptophan in to tryptamine. The biosynthesis of secologanin requires a number of enzymatic reactions of which the first step of reaction is the hydroxylation of geraniol to 10-hydroxy geraniol catalysed by the enzyme geraniol 10-hydroxylase ($G_{10}H$). Tryptamine and secologanin are condensed by the enzyme strictosidine synthase (SS) to form strictosidine, which is the common precursor of all indole alkaloids. This enzyme might be considered a logical point for limiting flux in the biosynthetic pathway.

The enzyme catalysing the condensation reaction was discovered and named by Stockigt and Zenk (1977). It was purified from *Catharanthus roseus* cell culture and characterized by Treimer and Zenk (1979). But the enzyme from *C. roseus* cell culture was found to split into four distinct multiple forms, which were difficult to characterize individually (Pfitzner and Zenk, 1988). Because of high yield of Strictosidine Synthase in *R. serpentine* as compared to *C. roseus* culture and the stability of soluble *Rauvolfia* enzyme (besides, the yield is also

considerably higher than that of *C. roseus*), the enzyme of *Rauvolfia* has advantage for its utilization in biotechnology. The time course of enzyme formation and growth parameters of cell in culture have been worked out. The enzyme is present with inoculum (day 6) in only small amount. The activity increases slowly to peak at day 12. In contrast, dry weight increase peaked at day 10 and maximal activity is therefore reached in the stationary growth phase.

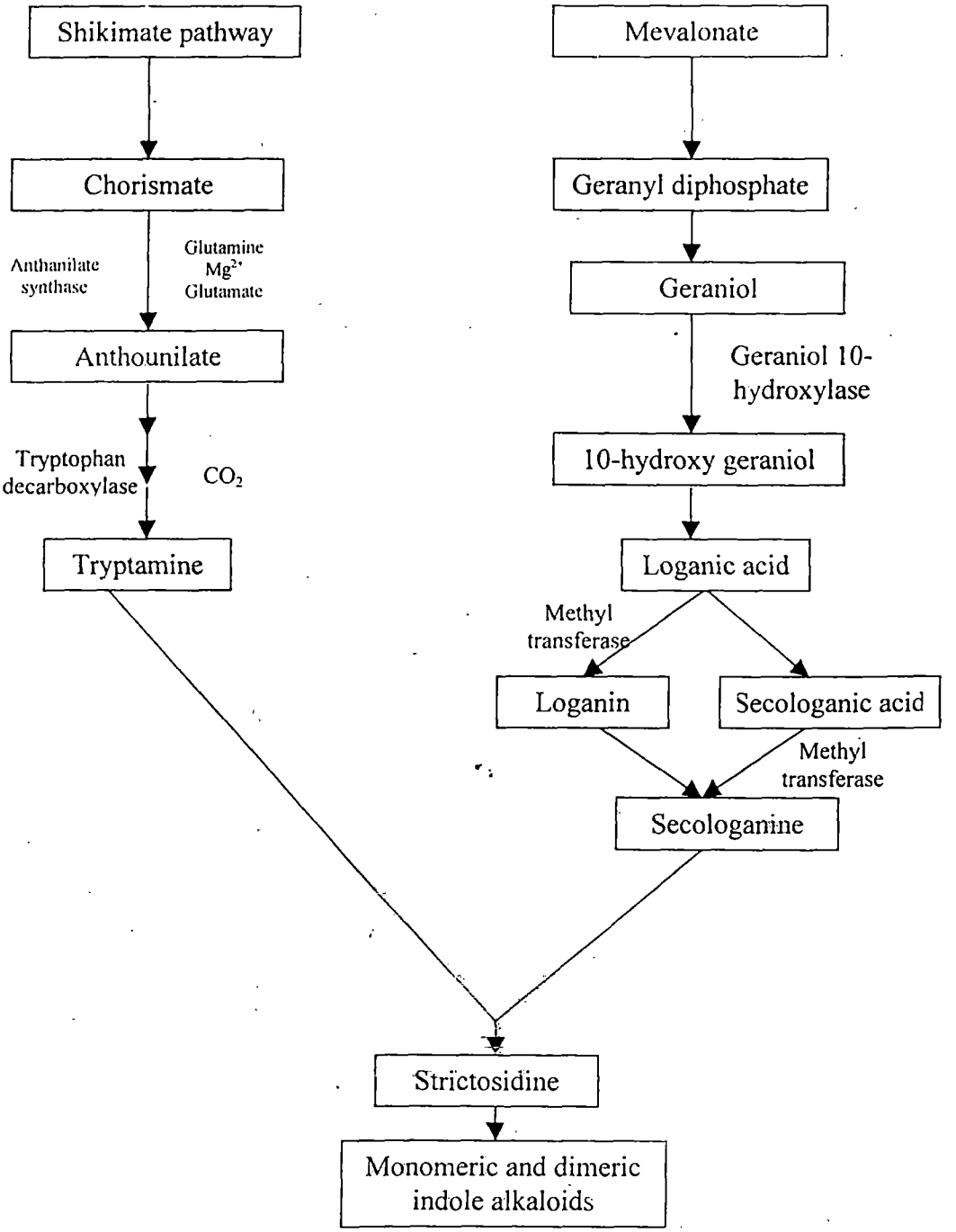


Fig. 1 : Biosynthesis of indole alkaloids (Misra *et al.*, 1996)

The availability of homogenous strictosidine synthase from *Rauvolfia* should facilitate basic studies regarding the structure and the application of the polypeptide.

In an attempt to study molecular biology of alkaloid biosynthesis and to learn about the organization of genes involved in secondary metabolism, it is necessary as a first step to purify strictosidine synthase to homogeneity. Because of the occurrence of multiple forms of synthase in *C. roseus*, the enzyme from *R. serpentina* is advantageous for its utilization.

Strictosidine was initially named as isovincoside. As an intermediate between isovincoside and cathenamine, 4,2,1-dehydrocornanthinealdehyde might be involved (Stockigt *et al.*, 1978)

Ajmaline synthase from *C. roseus* culture extract was made by Scott *et al.* (1977) but the enzyme rapidly lost its ability to synthesize ajmaline but retained glucosidase activity.

Meehan and Coscia (1973) isolated from *C. roseus* a microsomal mixed function oxidase, which convert monoterpene alcohol geraniol to their corresponding 10-hydroxy derivatives. This membrane bound cytochrome P-450 dependent monooxygenase is inhibited by an end product Catharanthine-an indole alkaloid suggesting feed back control of the pathway (Mc Farlane *et al.*, 1975), because both 10-hydroxy monoterpenes are good precursors for loganine. Stockigt *et al.* (1976) prepared an enzyme extract from cell suspension culture of *C. roseus* and could show the conversion of tryptamine plus secologanine in presence of NADPH into ajmaciline. If the incubation was performed in the absence of pyridine nucleotide, a new alkaloid cathenamine is accumulated and which is apparently an obligate intermediate in the biosynthesis of a ajmalacine and related alkaloids (Stockigt *et al.*, 1977).

Recently a new indole alkaloid of heteroyohimbine type was isolated from the roots of *R. serpentina* as ajmalicidine (Siddiqui *et al.*, 1987), but according to Lonnasmaa and Tolvanen (1994), it should be named as ajmaline and which was based on ^{13}C-NMR value. According to them, the methoxy-carbonyl group attached to the indole nitrogen is a very rare structural feature that was almost exclusively been found in certain *Kopsia* type alkaloid. The majority of the tryptophan derived alkaloid belongs to the class complex indole alkaloid which are built up from tryptamine and C_9-C_{10} unit.

It is now established that three general monoterpenoid skeletons are recognized as giving rise to most of the complex indole alkaloids; these skeletons are designed as the Aspidosperma, Corynanrhea and Ibago types taking the name of the plants which are rich in alkaloids with the respective monoterpenoid nuclei.

SUBCELLULAR COMPARTMENTATION OF INDOLE ALKALOID METABOLISM

The subcellular compartmentation of secondary metabolites is believed to play an important role in the regulation of their metabolism. Only a few enzymes in the pathway have been clearly localized. The subcellular localization of the enzymes involved in mevalonate pathway has two models. According to the first model, the mevalonate pathway occurs at three different sites, viz., cytosol, mitochondria and plastids. The second model suggests that the isopentenyl diphosphate is formed mainly in the cytosol and subsequently transferred to other compartments for synthesis of isoprenoids.

The shikimate pathway for biosynthesis of tryptophan is cytosolic. The conversion of tryptophan into tryptamine by tryptophan decarboxylase is also shown to occur in the cytosol. The product tryptamine is channelized into vacuole where strictosidine synthase enzyme is localized and, therefore, coupling of secologanin with tryptamine to form strictosidine occurs in vacuole.

The geraniol-10-hydroxylase is found to be associated with tonoplast (provacuolar membranes). It might be possible that the synthesis of secologanin occurs only in provacuole, while alkaloids are stored in mature vacuoles.

Ajmalicine is accumulated inside the vacuole by an ion trap mechanism. Ajmalicine freely diffuses across the tonoplast in neutral form and accumulates in its charged protonated form. Ajmalicine accumulation can be increased by acidification of the vacuole or by the proton pump activity. However, when there is a higher level of ajmalicine in the vacuole, it can convert into its tetra dehydro derivative serpentine by vacuolar peroxidases. The charged *serpentine* molecules are trapped inside the tonoplast. For ion trapping, energy flow comes from Mg^{2+} ATPase.

TISSUE CULTURE IN *Rauvolfia serpentina*

Mitra and Kaul (1964) grew excised embryos of *R. serpentina* in modified White's medium supplemented with IAA, 2,4-D, coconut water, yeast extract, adenine and adenine sulfate, either singly or in combination. Of the auxins tested for effectiveness in establishing root callus tissue in cultures, 2,4-D was found to be the most effective. Of different concentrations used 2,4-D at 1 ppm gave the best result. Excised embryos placed on a basal medium containing IAA (1 ppm) and coconut water (10%) responded in a different manner. From their hypocotyl and radical regions many roots were formed and their epicotyledonary apices developed into callus tissue. The three types of callus tissue isolated from the initial callus mass during successive transfers differed in their external and internal morphology, nutritional requirements, potentiality for differentiation and ability to synthosize reserpine. Callus tissue developed from the sides of young stem segments placed on the basal medium supplemented with IAA (1 ppm) and coconut water (10%), whereas 2,4-D and coconut water together failed to induce callus formation in stem segments.

Mitra *et al.* (1965) reported that callus formation was localized to the exposed cells at the cut faces of excised roots, stems and leaves and to the injured regions of cotyledons of *Rauvolfia serpentina*. During callus formation in excised root tips or radicale tips, the growth of the apical meristem was inhibited but the tissues of the subapical region continued to divide resulting in its swelling. The pith, phloem and cortex were induced to divide to form callus in radicale tips, excised roots and stems. The phloem and parenchyma around the vascular regions of the leaf lamina were activated to form callus in excised leaf segments, whereas palisade and spongy mesophyll remained inactive in the nutrient media used. Suitable media for rapid and continuous growth of these callus tissues were established either using modified nutrient solutions of White or Murashige and Skoog as basal media. Coconut water and 2,4-D were essential supplements to these media.

Mitra (1968) reported that growth of isolated roots of *R. serpentine* in a modified White's nutrient solution was enhanced when supplemented with α –NAA (0.001mg / l) and β-IBA (0.01mg/l); these factors did not prevent, after 21 days of culture, a decline in growth rate. By successive transfer of 21 days old culture, isolated roots continued their growth through 4 passages. Kinetin (0.001 mg /l) slightly enhanced the level and duration of growth of excised roots. The roots in light grown cultures receiving kinetin became pale green. Gibberellic acid at a concentration of 0.01 mg /l enhanced linear growth of main axis in the first passage only in dark cultures. Casein hydrolysate (vitamin free, DIFCO) and ammonium nitrate were not beneficial to growth of isolated roots.

Nikolaeva *et al.* (1978) achieved the production of a finely dispersed suspension culture of *R. serpentina* tissue by collection and transplantation of only fine aggregates and isolated cells. Examination of the characteristics of the aggregated and accumulated alkaloids in the suspension culture revealed no differences in content of alkaloids in aggregates of different dimensions. The method of collection and transplantation of only fine aggregates and individual

cells of suspension cultures made it possible to produce suspension cultures relatively homologous in their morphology.

Vollosovich and Vollosovich (1982) studied the possibility of cultivating *R. serpentina* on microbiological waste, such as molasses with 50% sucrose, hydrols from lignin and corn with 40% glucose, as well as syrup and green syrup, was studied. Molasses and hydrols could not be used to cultivate *R. serpentina* because of their toxicity. The green syrup was promising for the cultivation of isolated plant tissues. The syrup could be used in industry to obtain *R. serpentina* biomass.

Vollosovich *et al.* (1982) reported that the optimum of alkaloids in the tissue culture of *R. serpentina* was obtained at 1:60 ratio of ammonium nitrate and sucrose in the nutrient medium. Increase in ammonium nitrate concentration decreased tissue weight, enhanced alkaloid and protein synthesis and suppressed starch accumulation. Higher concentrations of sucrose acted in the opposite direction.

Pfitzner *et al.* (1984) isolated a plant enzyme vellosimine reductase from *Rauvolfia* cell suspension cultures. This new enzyme was purified (110-fold) and characterized. The reductase is a specific enzyme of the sarpagine pathway catalyzing the NADPH dependent conversion of vellosimine into 10-deoxysarpagine. The latter alkaloid is the immediate biogenatic precursor of sarpagine as shown by its high in vivo incorporation rate (86%) into sarpagine.

Uesato *et al.* (1984) administered 2H and 13C labelled compounds to *R. serpentina* suspension cultures and the results indicated that ajmaline and vomilenine produced by these cultures were biosynthesized via 10-hydroxygeraniol, 10- hydroxynerol and iridodial in the same way as secologanin, vindoline etc. in *Catharanthus roseus* and *Lonicera morrowii.* Therefore, this cyclization mechanism appeared to be common in plants containing secoiridoids and indole alkaloids.

Akram and Ilahi (1985) reported that callus cultures of *Rauvolfia serpentina* were initiated on MS medium supplemented with 1% casein hydrolysate, 1mg/l NAA and 0.5 mg/l kinetin. Stem callus, which regenerated buds, was heterogeneous in texture, friable at the periphery and top but compact and hard at the base and core. Bud regeneration was noticed after 20 weeks culture of callus on White's root culture medium containing 100 ml/l coconut milk, 10 ml/l biotin, 250 mg/l sodium diethyl-dithiocarbamate and 0.8 mg/l NAA. The plantlets formed roots both with 0.8 mg/l NAA given continuously or with 24 h. treatment of 3 mg /l each of IAA and IBA. The plantlets established themselves quite easily in soil. For the first few weeks in soil they were nourished with half strength Knop's solution. Afterwards they became completely autotrophic.

Roja *et al.* (1985) isolated and characterized some indole alkaloids from multiple shoot cultures of *R. serpentina*. Shoot cultures were established from the axillary meristem of the field –grown plants. The cultures were maintained on MS agar medium supplemented with 0.1 ppm of NAA and 1.0 ppm of BA. The shoot cultures were dried and extracted with hexane followed by ammonia / methanol. Identification of the alkaloids was achieved through TLC, mass spectral analysis and HPLC. The alkaloid pattern (TLC) of the shoot cultures, leaves and roots differed considerably. The shoot cultures contained alkaloids of the roots (ajmaline, yohimbine) as well as the alkaloids of the leaves (ajmalidine). Colorimetric estimation of the total alkaloids indicated that the shoot cultures contained 0.71%, the leaves 0.54% and the roots 2.64%.

Akram and Ilahi (1986) reported that using benzyl aminopurine (BAP) (2 mg/l) and NAA (0.8 mg/l) root callus of *Rauwolfia serpentina* was induced to bud formation which further developed into shoots. The isolated shoots rooted with 24 h treatment of 3mg/l IAA and 3 mg/l IBA. The plantlets transferred to soil were initially watered with half strength Knop's solution until they became autotrophic and were found to grow well in open field conditions. The root callus is, therefore, suggested as a potential tissue for the formation of plantlets in *R. serpentina.*

Schubel *et al.* (1986) isolated a novel highly substrate specific *Rauwolfia* enzyme, raucaffricine beta-D–glucosidase, from cell suspension cultures of *R. serpentina*. The enzyme was purified and its major characteristics were investigated. Its limited distribution in different cell cultures and differentiated plant revealed that the enzyme was present in significant amounts exclusively in cultured Rauwolfia cells.

Yamamato and Yamada (1986) maintained cultured *R. serpentina* cells on a modified MS medium for 13 years and these cells produced much more of the pharmecologically important alkaloids-ajmalicine (0.005-0.12%) and reserpine (0-0.003%)

Ilahi and Akram (1987) observed that young leaves of *Rauwolfia serpentina* inoculated on MS medium supplemented with NAA (1 mg/l) + Kinetin (K) (10 mg/l) and 10% coconut milk under 24 h. light induced callus, while 2,4-D induced callus under 16 h. light. Callus induction and its growth was more on explants taken from *in vitro* raised seedling on 2,4-D (1mg /l) or NAA (1mg/l) +K (10 mg/l) and 10% coconut milk under 24 h light. On sub-culturing the callus exhibited good growth. Similarly, leaf callus growth on Abou Mandour (AM) medium containing NAA, BAP, K, adenine sulphate and 2,4-D with 1g/l of casein hydrolysate showed excellent growth. The cultures analyzed showed 0.00686, 0.00489 and 0.00735% serpentine in calli growing on MS medium with NAA + K, 2,4-D and AM medium respectively. In the same cultures ajmaline was found at 0.0249, 0.0281 and 0.040%, respectively.

Yamamoto and Yamada (1987) reported that the cultured *R. serpentina* calluses consisted of cell colonies that had different fluorescences under 365 nm UV- light. These were divisible into two main categories, yellow-green and blue-white. The HPLC analysis had shown that the yellow-green fluorescent strains produced much reserpine, while the other variety produced much of 3,4,5-trimethoxybenzoic acid. A combination of 10M BA and 10M NAA enhanced production of reserpine in the yellow-green fluorescent variety.

Mathur *et al.* (1987) developed a tissue culture procedure for the establishment and propagation of a colchi-autotetraploid of *Rauvolfia serpentina* for possible commercial exploitation. Multiplication of autotetraploid shoots was obtained either through axillary bud elongation on MS medium containing 0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin, via multiple shoot formation on MS medium supplemented with 1.0 mg l^{-1} 6-benzyl amino purine and 0.1mg l^{-1} NAA. Rooting was induced by transferring the shoots to MS medium containing 1.5 mg l^{-1} NAA alone. The plantlets, thus formed, were tetraploid in nature by cytological observations of the root tips. They exhibited 80-90% success in establishment under glasshouse and field conditions.

Hampp and Zenk (1988) purified strictosidine synthase to homogeneity from suspension cells of *Rauvolfia serpentina* (920-fold purification, 35% yield). This enzyme which catalyses the stereospecific condensation of secologanin and tryptamine to H-3α (S)-strictosidine, is the key intermediate in monoterpenoid indole alkaloid biosynthesis. The specific activity was184 nkat/mg. The isolated enzyme was a single polypeptide, Mr 30000, possessing a 5.3% carbohydrate content. The enzyme had a pH-optimum at 6.5, a temperature optimum at 45°C, isoelectric point at pH 4.5, and apparent K_m values both for tryptamine and secologanin of 4 mM. The enzyme was immobilized and had, in this form, a half-life of 100 days at 37°C.

Ilahi *et al.* (1988) used various organs such as root, leaf and stem of *Rauwolfia serpentina* to induce callus formation and then organogenesis. Plantlet regeneration was achieved in root and stem calli by a combination of different growth hormones viz., NAA, IAA, IBA, BAP, kinetin. For this different media viz., White's Root Culture (WRC), Murashige and Skoog (MS) and Abou Mandour (AM) were utilized. Hormonal requirement differed with the explant source. A large number of plantlets was produced *in vitro* for further establishment under the natural conditions. Root, stem and leaf calli were analyzed for alkaloids. Ajmaline was the major alkaloids produced by cultures.

Alkaloids in such plants were higher in leaf and stem cultures than the parent plant.

Ilahi and Khan (1988) obtained root explants from mature plants and sub cultured juvenile aseptic seedlings for callus formation with 2,4-D, NAA and kinetin. Explants taken from seedling roots gave the best response for callus formation or WRC medium containing 1 mg/l 2,4-D and 100 ml/l coconut milk. Callus propagation in subcultures was found good both on initiation medium and on AM medium with BAP, K, AS, NAA, 2,4-D and caesine hydrolysate (CH) at 0.1, 0.3, 0.4, 1.0, 6.0 and 1000 mg/l. The alkaloids screened were serpentine, ajmaline, raubasine, raupine and reserpine. The major alkaloid present in cultures was ajmaline. Its maximum percentage was 0.0573% in the cultures grown under dark on AM medium, corresponding to an increase of 94.61% over cultures kept for 16 hr. in light.

Akram *et al.* (1990) observed the behavior on of field trials of root regenerated plants of *R. serpentina*. Root callus was induced to differentiate buds with 0.8 mg l^{-1}NAA and 2 mg l^{-1} benzyl adenine purine (BAP). Buds were rooted with 24 h. treatment of 1AA + 1BA (both 3 mg l^{-1}). Rooted buds were differentiated into autotrophic plantlets. The plants on transfer to soil thrived well and matured successfully to produce flowers and fruits. Karyotype analysis of flower buds showed pollen mother cells as having 22 chromosomes (2n), as in cultivated *Rauwolfia* plants.

Roja *et al.* (1990) reported that the multiple shoot cultures of *R. serpentina* were established from axillary meristems of the field grown plants on MS medium supplemented with BA (1.0 ppm) and NAA (0.1 ppm). Growth hormones influenced the morphogenetic events of the shoot cultures, such as root initiation in 1AA combinations, stunted shoot formation in 2,4-D and slender shoot formation in kinetin + NAA combinations The morphogenetic responses were associated with marginal changes in alkaloidal production. The cultures produced high levels of alkaloids including ajmaline (0.15%), ajmalidine and 3-epi-α-

yohimbine. The alkaloid concentrations in the cultures were comparable to the alkaloids in the roots of the intact plant.

Jocelyne and Cheniux (1991) cultured *R. vomitoria* mesophyll protoplasts in Murashige and Tucker liquid medium containing growth regulators. Calli produced shoots, however, rooting did not occur. Somatic embryos achieved different patterns of development.

Ruyter *et al.* (1991) compared the indole alkaloid content of *Rauwolfia serpentina* roots from regenerated plants (from stem and root callus) with the parental stock. Although the total alkaloid content appeared to be slightly higher in the roots from the regenerated plants, HPLC- analysis of individual alkaloids indicated that the contents of the alkaloids ajmaline, serpentine and reserpine were lower than in the roots of the parental stock. The glucoalkaloid raucaffricine was identified as a constituent of all samples, thus providing the first evidence for its occurrence in roots of *R. serpentina.*

Sharma and Chandel (1992) reported that on a standard shoot culture medium, nodal cultures of *R. serpentina* could be maintained for nine months at 25°C. Low temperature incubation of *in vitro* cultures appeared highly promising as cultures exhibited normal health even after 15 months of storage at 15°C. On the other hand, 10°C and 5°C were found deleterious to growth of *the R. serpentina* cultures.

Roy *et al.* (1995) studied *in vitro* culture method for the clonal propagation of *Rauvolfia serpentina.* They used shoot tips and lateral buds from field-grown plants as explants. When the explants were cultured on MS medium with 1.5 mg/l BAP + 0.5 mg/l NAA, multiple shoot buds were formed. Subculture in the same nutrient medium gave a higher number of shoots. When the regenerated shoots were excised and subcultured individually in the same nutrient medium, they also produced multiple shoots. The shoots had continued to proliferate through ten subcultures with average 25 shoots per transfer. For rooting, the shoots were

excised from the culture flask and implanted individually on root induction medium consisting of half strength MS salts supplemented with 1.0 mg/l each of IBA and IAA. Within 3 weeks of transfer 100% rooting was achieved on this medium. They were transferred to a tray containing soil and compost and covered by polyethylene sheets. After 2 weeks they were transferred to the open field where 95% of the plantlets survived.

Sharma *et al.* (1995) reported procedure for in vitro multiplication and *in vitro* conservation of six threatened endangered medicinal plants viz., *Colcus forskohilii, Gentiana kurroo, Picrorhiza kurroa, Rauvolfia serpentian, Saussurea lappa* and *Tylophora indica*, Various combinations of growth regulators were tested to select optimal medium for initiation and further shoot multiplication. Slow growth experiments were performed and the shelf-life of shoot cultures in multiplication and/or modified medium could be extended for 11-20 months depending on the species.

Sarker *et al.* (1996) reported that multiple shoots were induced from nodal segments and shoot apices *of Rauvolfia serpentina.* MS medium containing 1.0 mg/l BA and 0.1 mg/l NAA was found to give the best shoot proliferation rate. Callus formed at cut bases of the explants which produced shoots when sub cultured on media containing low concentration of BA (0.5 or 0.1 mg/l) and NAA (0.1 mg/l). The regenerated shoots were treated for 10 days in NAA or IBA supplemented media and then transferred to auxin omitted media for root initiation. Of the two auxins tested, NAA was found to be more effective than IBA and maximum rooting (83%) with 4-8 roots per shoot was recorded on the medium containing 1.0 mg/l NAA. The plants with well-developed root systems were transferred to pots containing soil and sand mixture and nearly 60% of the plant survived.

Ilahi *et al.* (1997) conducted tissue culture experiments using nodal explants on MS medium supplemented with various phytohormones. A pinkish-yellow callus appeared on nodal segments cultured on MS fortified with 1.5 mg/l

BAP and 10 mg/l 2,4-D. The induced callus could be easily proliferated on the same medium. Excellent callus was also induced when MS was supplemented with 0.5 mg/l each of IAA and BAP. Prolific shoot formation occurred on this callus when BAP and IAA were added at 1.0 mg/l each along with 5.0 mg/l adenine sulphate (AS). Shoot regeneration frequency could be further increased by culturing this organogenic callus on MS medium fortified with 1.0 mg/l BAP and 5.0 or 10.0 mg/l AS. This callus could be maintained on medium of the same hormonal concentration for an indefinite culture period without any obvious loss in its vigour. Roots could also be regenerated on the organogenic callus cultured on BM fortified with 1.5 mg/l Kn and 1.0 mg/l 2,4-D. Rooting of the young shoots occurred on a medium containing 1.0 mg/l each of IBA and NAA and 2.0 mg/l 2,4-D. These plantlets were hardened and transferred to field condition. All the important *Rauwolfia* alkaloids have been isolated from the callus, regenerated shoots and roots using various techniques. The alkaloid concentrations in the cultures were compared to those in the root of intact plant.

Recently Akhtar *et al.* (2001) has reviewed the hairy root culture of medicinal plants including *R. serpentina.*

SEED GERMINATION, VEGETATIVE PROPAGATION AND CULTURE OF DIFFERENT SPECIES OF *Rauvolfia* WITH SPECIAL INTEREST ON *R. serpentina.*

Abrol *et al.* (1956) carried out an experiment on the method of propagation of *R. serpentina* in Jammu and Kashmir state of India in March-April period. Seeds were procured from South India and were pretreated with different concentrations of H_2SO4 before sowing in the months of June and July. It was found that, treatment by 90% H_2SO4 for about 1 min gave as high as 60%-70% germination and seeds were germinated in about 3 weeks time.

Biswas (1956) made an effort for the experimental cultivation of *Rauwolfia serpentina* and *R. canescens* in the lower hill-ranges of Rongoalong, the border of Bhutan. The average rainfall at the place was 273 mm at an elevation between 1500 ft. and 38000 ft., temperature varied from maximum 87°F to minimum 46°F with high humidity. The highest percentage of rooting of cuttings after one month was 81.25 in June and 62.5 in May. The average percentage of rooting of cuttings planted from March to June was 58. The germination of the seed, however, varied from March to July was March-45 days 10 to 15%, April-34 days 15 to 20%, May-21 days, 25 to 30%, June-38 days 10 to 15% and July-48 days 5 to 10%.

Badhwar *et al.* (1956a) found a wide difference in percentage of germination of *Rauwolfia serpentina* Benth-seeds, and to find out the optimum temperature and humidity for their germination. They already collected some data on temperature and rainfall in Dehra Dun, India from March to July and their effect of raising the crop through root cuttings and stem cuttings during this period.

Badhwar *et al.* (1956b) studied the methods of propagation and their effect on root production in *R. serpentina.* The comparative performance of plants raised by different methods indicated that the sub-aerial portion and the root were the best in those raised from seeds. It was about 6.5 times that of roots produced by plants raised from stem cuttings and 3.5 times that of roots obtainable from plants grown from root cuttings. By planting small stumps of roots with a portion of the stem above the collar resulted in 100% success.

Sobti *et al.* (1956a) worked on the cultivation of *R. serpentina* in an experimental scale in different states of India. Classified seeds obtained from 8 sources by the 'Float and Sink' method into (1) light seeds-floating on water, (2) medium seeds-sinking in water but floating on 10% NaCl solution and (3) heavy seeds; sinking in 10% NaCl solution. It was found that germination was 4% in case of seeds floating on water, the percentages of germination was greatest

(about 20%) in seeds which sink in 10% saline solution, and floated on saline but sinking water was nearly 12%.

Sobi *et al.* (1956b) reported about the analysis of roots of one year, two years and three years old plants for their total alkaloids in *R. serpentina* was found that 1 year old plants had 1.6% which was slightly less than that of two and three years old plants had 1.7%. These results indicated that *R. serpentina* can be grown in Jammu and Kashmir state in India and its large scale propagation from seeds is possible provided the seeds are properly selected.

Hedayatullah (1959) reported a detailed study regarding the culture and propagation of *R. serpentina.* For the vegetative propagation of the plants, stem, root and leaf cutting were experimented with. It was found that both stem and root cuttings could be used successfully for the vegetative propagation, but leaf cutting had no propagative value. However, with seeds, among the freshly ripened fruits, dried fruits and peeled seeds, the peeled seeds were most suitable for propagation. Higher average percentage germination was obtained during the four months of the monsoon, i.e., May, June, July and August. The spacing of 1½' X 1' was most suitable for cultivation of *R. serpentina.*

Dutta *et al.* (1963) reported the different aspects of cultivation of *R. serpentina.* This plant could be propagated by seeds, root cuttings, stem cuttings and root stumps. A success of 51 to 82% was observed with root cuttings. 40 to 65% success was obtained by stem cuttings. However, better results were obtained after applying 40 ppm solution of IAA. Outstanding success of 90 to 95% survivals was met with root stumps. The limitation of this method in extensive plantations was that it required a large number of root stumps. Among these methods of propagation, optimum yield was obtained when propagation was by seeds. It was found that there was no significant increase in the yield of roots as a result of the application of fertilizers. In some cases, slight increases were recorded with superphosphate. Spacing of 45 cm between the rows and 30 cm

between the plants was recommended. It was most economical to harvest roots after 15 months of planting.

Mitra (1976) studied the factors responsible for the formation of non-viable and viable seeds of *R. serpentina*. Inflorescenes were ontogenetically axillary. Flower primordia developed up to the stage of opening of flowers in 30-35 days and into mature fruits in another 40-45 days. Pollen grains were highly fertile which could not be a cause for formation of non-viable seeds; pollen and stigma were found compatible as shown by high percentage of fruit setting in self-pollinated flowers. Hardly any seed without embryo and endosperm was found, seeds of fully mature black fruits were of 2 kinds, one with mature embryos enclosed in solid endosperm and the other with immature embryos within shriveled and desiccated endosperm. Non-viability of seeds was due to shriveling up of endosperms, which in turn arrested the development of immature embryos to become mature. Degree of temperature and percentage of humidity prevailing in different months caused shriveling up of endosperm at its milk stage to form varying number of non-viable seeds in different months. Harvesting of ripe black fruits in May gave the highest percent (85) of viable seeds and the lowest percent (15) of non-viable seeds.

Maheswari *et al.* (1982) tested germination of two varieties of *R. serpentina* seed, collected from Regional Research Laboratory Jammu-Tawi (JT) and Araku Valley (AV) of Vishakhapatham. Out of 128 seeds of JT variety, 110 seeds (86%) germinated, whereas out of 120 seeds of AV variety, 64 seeds (53.3%) germinated. Use of fresh seeds in place of stem/root cuttings for viable propagation was suggested.

Mohammad and Shukla (1986) reported that the use of pesticides was not suitable for maximal sprouting from roots cuttings of *R. serpentina*. Nutrient carriers combined with growth hormones were useful in hastening up the sprouting and emergence of healthy plants.

Chandra (1956a) took hand wood cuttings of *Rauwolfia canescens* L, 12 to 15 cm in length and varying diameter (0.4 to 1.0 cm) and treated them with 30 ppm solution of Indole acetic acid and naphthalene acetic acid for 12 hours. Controls were similarly treated with water only and all were planted in pots with sand under irrigation. It was observed that in the beginning all the cuttings gave out new leaves. For more than one month all sets including controls had green leaves, but later the leaves of controls started wilting. The leaves of treated ones continued to be green. After one month and a half the treated cuttings started production of callus. The observations were continued further till no more mortality was observed.

Chandra (1956b) further reported that *R. serpentina* preferred clayey soils. In the neighbourhood of Lucknow, *R. serpentina* plants were found to flourish well under mango trees. In view of this, it was suggested that cultivation could profitably to be carried in mango orchards, which provide sufficient shade and other suitable condition for the best growth of *R. serpentina*.

GROWTH PHYSIOLOGY OF *Rauvolfia serpentina* UNDER VARIOUS TREATMENTS AND CONDITIONS WITH SPECIAL INTEREST ON THEIR PRODUCTIVITY OF ALKALOID CONTENT

Hedayatullah (1959) reported that the economic harvesting age of *R. serpentina* in respect of yield per acre and alkaloids content was between 12 months and 18 months after transplantation. It was observed that the yield of root varied between 1500 lbs and 300 lbs per acre.

Siddiqui *et al.* (1959) reported that different plant growth factors–both external and internal may affect the alkaloids content of the plant. Age of the routine of *Rauwolfia serpentina* had influence on the alkaloidal content. The young roots had relatively lower alkaloids content than that of two years old plant.

Dhar (1965) studied the variation in the alkaloid content and morphology of four geographical races of *R. serpentina*. These races were collected from Rangu, Rishikesh, Calcutta and Dehra Dun. The plant collected from Rishikesh was morphologically different from all other races. The alkaloid content was highest in Rishikesh race (2.66%) being double than that of the Calcutta race which contained 1.35 %, Rangu and Dehra Dun races were intermediate. The results suggested that it would be possible to select high alkaloid-containing races of *R. serpentina* for large scale cultivation.

Biswas (1969) studied the growth and changes in the alkaloidal contents of *R. serpentina* under complete defloration treatment. Plants under complete defloration were bushy in form as a result of intensive vegetative growth, both in terminal and lateral shoots, which caused proportionate increase in root growth. The roots were more branched with profuse production of fibrous roots compared to control. Complete defloration resulted in large increase (62.9% in fresh and 56.50% in moisture free root, respectively) of root weight. An appreciable increase (18.00% based on moisture free root) of total alkaloid content per unit weight of the moisture free root was also observed.

Saini and Mukherjee (1970) reported the effect of complete and partial defloration on the root yield of *R.serpentina* under low and high levels of nitrogen fertilization. Reproductive growth, even when partially reduced, limited root growth. Extra application of nitrogen resulted in an increase in reproductive growth but was without any effect on root yield. A complete defloration increased root weight and also caused the plants to respond to extra application of nitrogen. The onset of dormant period was delayed by about a month under complete defloration. The increase in root weight was due to a proportionate increase in stem and leaf growth caused by defloration treatments. The alkaloid content per unit weight of dry root did not change with complete defloration.

Biswas (1970) also reported the results of four treatments of defloration (including control) with two nitrogen levels on total yield of root and alkaloid

content of *R.serpentina*. Defloration treatment consisted of partial defloration in which each of the inflorescences was cut retaining 10 or 5 flowers. The results revealed that a substantial increase in the moisture free weight of the root was due to complete defloration. The increase was more marked at high nitrogen level. In the partially deflorated plants, the effect of defloration was non-significant even at high nitrogen level. There was also a substantial increase in the alkaloid extent with complete defloration, at both levels of nitrogen. No such differences were noticed for partial deflorations.

Biswas (1971) observed the effect of complete defloration treatment on the differentiation of wood and bark of the root and total alkaloidal contents of *R. serpentina*. Complete defloration resulted in increased production of moisture-free intact root, root wood and root bark to the extent of 28.20, 20.26 and 38.80% over those of the control. An increase in the total alkaloid contents (0.87, 0.01 and 0.80% of moisture-free intact roots, root woods and barks, respectively) was also observed in the roots of plants under complete defloration. The root wood and bark ratio was noticed to be lowered in completely deflorated plants, which produced much thicker barks as compared to the root wood.

Later Biswas (1973) made an investigation to study the effect of complete defloration on the growth and development, yield of moisture-free root and shoot and changes in the alkaloidal (total, reserpinoid and residual) mineral (N, P and K), sugar (total, glucose and sucrose) and ash contents of roots of *R. serpentina*. Complete defloration had a profound influence on the growth and development. The increase in the total yield of moisture-free roots per plant under complete defloration was intimately associated with the appreciable increase in the total, reserpinoid and residual alkaloids per unit of moisture-free root. The increased sugar and mineral contents had direct bearings of the production of the alkaloids.

Nandi and Chatterjee (1975) made an attempt to increase the total alkaloid yield of *R. serpentina* by N:P:K trials either singly or in combination. It was found that alkaloid biogenesis in root remained inversely correlated with laminar area.

Those treatments, which distinctly reduced lamina area, enhanced the total alkaloid content. Maximum alkaloid synthesis was noticed in combination with N:P:K and this increase in the content of total alkaloid, gained momentum with the advancement of age.

Nandi and Chatterjee (1978) also studied some of the very important areas of growth and physiology of eleven medicinal plants including *Rauvolfia serpentina* with special reference to synthesis of active principles in relation to light and growth hormones. In *R. serpentina,* enhancement of alkaloid synthesis had been revealed in higher photoperiodic cycles only. In the species, post-reproductive stage showed enhanced aklaloid synthesis and increase was about 7% over the vegetative stage. GA_3 treatment also promoted the synthesis of alkaloid, suggesting that the mechanisms of biogenesis of alkaloid were equally sensitive towards GA_3 as well as light.

Biswas (1982) evaluated correlation at the genotypic level between some characters and root yield and alkaloid content in two populations of *R. serpentina.* The root yield showed highly positive correlation with branch per plant in the clonal population, serving as a good indicator of root production. A significantly positive correlation was observed between root yield and alkaloid content. Clonal population showed better association between different characters than the seedling population.

Maheshwari *et al.* (1985) reported that at both Indore and Akola, the net returns were higher when *Rauvolfia* was intercropped than when grown alone. At Indore, soybean in the wet season and garlic in winter were most suitable and remunerative intercrops followed either by chilli in the wet season and onion in winter or soybean in the wet season and onion in winter. *Rauvolfia,* soybean and garlic in association gave the highest total net returns per hectare (Rs. 37,952), followed by *Rauvolfia,* chilli and onion association (Rs. 36, 538) and *Rauvolfia,* soybean and onion association (Rs. 35,917). At Akola, soybean in the wet season followed by onion in winter was the most remunerative intercrop (Rs. 28,630/ha),

followed by *Rauvolfia*, soybean and garlic combination (Rs. 26,360/ha) and *Rauvolfia*, chilli and onion association (Rs. 26,011/ha). The quality of the roots of intercropped *Rauvolfia* was comparable to pharmacopoeial standard.

Haqh *et al.* (1986) treated *Rauvolfia serpentina* plants with 500 and 1000 ppm potassium napthenate (KNap) to study the effects on a number of vegetative, reproductive and biochemical parameters. Treatment with 500 ppm KNap caused little or no effect on any of the parameters studied. However, treatment with 1000 ppm KNap resulted in significant increase in plant height (26%), number of leaves (36%), leaf area (29%), number of branches (63%), number of inflorescences (84%), number of fruits (75%), total alkaloid content of roots (23%), total alkaloid content of leaves (5%) and reserpine content (11%), ajmalicine content (6%) and sterol content (3%) of the roots.

Maheshwari *et al.* (1988) studied the effect of N and P fertilizers on the yield of roots, its alkaloid content and alkaloid yield of *R. serpentina*. An increase in the level of N greatly increased dried root yield (maximum at 45 kg N/ha). The total alkaloid content in root and its yield remained higher at 30 kg N/ha followed by 45 kg N/ha compared with the control. An increase of the level of P appreciably increased the dried root yield. The maximum was recorded at 60 kg P/ha, beyond which it declined. The total alkaloids and their yield were higher at 60 kg P/ha, the biometric traits such as plant height, dried root weight, dried root: shoot ratio and diameter of root increased with N and P by 45 and 60 kg/ha, respectively. These two levels also gave higher net monetary return.

Maheshwari *et al.* (1991) conducted an experiment to schedule the irrigation for *R. serpentina* on a shallow black soil. The irrigations were given on the basis of cumulative pan evaporation (50 mm) at irrigation water: cumulative pan evaporation ratio of 0, 0.15, 0.30, 0.45, 0.60, 0.75 and 0.90. The dry root and alkaloid yield and water use efficiency were increased upto an IW: CPE ratio of 0.75. The alkaloid content was not affected by irrigation schedules. The maximum net returns/ha were obtained at an IW: CPE ratio of 0.75.

Sethi *et al.* (1991) studied the variation of chemo-botanical characters in the indigenous collections of *R. serpentina.* The highest range of variations for total root weight/ plant and number of secondary roots/ plant was observed in the collections from Coondapur and Conacana regions of Karnataka and Goa, respectively. These two characters contributed to high alkaloid recovery. The Coondapur region gave the highest range (1.58–2.03) of total alkaloids with of mean of 1.81. The same pattern was observed for reserpine content with the range of 0.07-0.24 and mean value of 0.16%. Such chemo-botanical variations in these materials were natural due to their geographic, ecological and topographic variation.

CHAPTER 2

ISOLATION, PURIFICATION AND CHARACTERISATION OF RESERPINE FROM ROOT OF *Rauvolfia serpentina* WITH SPECIAL INTEREST ON THE ESTABLISHMENT OF AN EASY AND RAPID COLORIMETRIC METHOD FOR ITS QUANTITATIVE ESTIMATION

INTRODUCTION

The dried root of *Rauvolfia serpentina* commonly known as serpentina root, in Sanskrit as sarpagandha and in Hindi chandrabhaga is one of the most important drugs used in modern medicine. Once it was a commonly accruing local plants in the plains of Darjeeling District but due to ruthless collection from the natural habitat it has faced endangered condition. This plant is commercial source of reserpine, an indole alkaloid, which is one of the most important therapeutically useful drug.

The importance of Rauvolfia serpentina in modern medicine was recognised in 1952 when Muller succeeded in isolating pharmacodynamic principle reserpine in the roots of the plant which revolutionized the therapeutic use of the drug as antihypertension and sedative. More than 20 different indole alkaloids have been isolated so far from this plant and most of the alkaloids are confined to the roots of the plant. The total alkaloid content in the root range from 1.7 to 3.0 percent which are mostly concentrated in the bark (about 90%). Alkaloids are also present in leaves, stem and seeds but not in significant amount as compared to roots. Siddiqui and Siddiqui (1931, 1935) isolated reserpine and other indole alkaloids at the very beginning when the importance of Rauvolfia

serpentine was known. They were the pioneer workers in this line of investigation, but they followed cumbrous method. They collected different solvent parts following the method of fractional separation. Each fraction contained a mixture of alkaloids and the modern method of chromatography was not used to separate pure alkaloids very easily.

Besides most of the methodologies so far recorded in connection with the quantitative estimation of reserpine have been observed to involve costly instruments, which are generally not available in most of the laboratory. More over all these involved cumbrous process without the consideration of purification of reserpine.

Thus an attempt has been made to work out an easy and rapid colorimetric method for quantitative determination of reserpine involving the newly developed methodology for purification of the natural product.

MATERIALS AND METHODS

MATERIALS

Root bark of *Rauvolfia serpentina* was collected from Sukna, Mirik area.

METHODS

Extraction : 500 gms of dry powdered bark of *Rauvolfia serpentina* was extracted using soxhlet apparatus with chloroform for 5 hours per day and continued for 3 days. The extracted chloroform part was separated and evaporated to dryness.

Purification under column chromatography: A glass column was packed with first absorbent cotton plug then alumina (Al_2O_3) in wet condition in

petroleum ether. The concentrated mass of chloroform extract was absorbed. Various following solvents and their mixtures were eluted one after another as represented below:

Solvents/ solvent mixtures	**Ratio**
Petroleum ether	Pure
Petroleum ether : Benzene	(3:1)
Petroleum ether: Benzene	(1:1)
Petroleum ether: Benzene	(1:3)
Benzene	Pure
Benzene: Chloroform	(3:1)
Benzene: Chloroform	(1:1)
Benzene: Chloroform	(1:3)
Chloroform	Pure
Chloroform: MeOH	(3:1)
Chloroform: MeOH	(1:1)
Chloroform: MeOH	(1:3)
MeOH	Pure

Various fractions after elution were collected in a conical flask and evaporated to dryness on electrically operated hot plate. Various solid materials obtained after elution were subjected to paper chromatography for identification of chemicals.

Identification of chemicals by paper chromatography: A piece of chromatography paper (Whatman no. 1) was taken and concentrated mass of

chemicals dissolved in chloroform was spotted on the paper along with the authentic marker and ran under different solvents or their mixtures in ascending type of chromatography. After running of chromatography for desirable time when solvent front has run for a considerable length. The paper was taken out and dried under hot air woven and it was kept in iodine chamber so the area containing chemicals became dark in colour and then Rf was calculated.

Determination of melting point: Fine crystals were taken in a capillary tube blunt on one end it was tapped so that the crystals accumulated at the bottom of the capillary tube and melting point was determined using electrically operated melting point apparatus.

Detection of alkaloid under UV light: The paper containing the Rauwolfia alkaloids were exposed under UV light in a dark room. The spots containing Rauwolfia alkaloid was detected by green fluorescence.

Identification and characterisation of alkaloid by IR spectrum: The isolated chemical was identified and characterised to be reserpine after comparing the absorption peak in IR spectrum of isolated product with those of authentic sample.

Preparation of stock solution of reserpine: Authentic reserpine (Sigma company) of 10 mg was dissolved in 0.5 ml of chloroform and acetic anhydride was taken to make it a volume of 10 ml to make 1000 ppm of stock solution.

Preparation of different grades of stock solution: From 1000 ppm of stock solution different grades of 900, 800, 700, 600, 500, 400, 300, 200, and 100 ppm were prepared by dilution with acetic anhydride.

Preparation of reaction mixture: 1 ml of reserpine solution in acetic anhydride was mixed with 1 ml of concentrated sulphuric acid (H_2SO_4). It was shaken properly and kept for 10 minutes and then brown colour appeared.

Determination of absorption maxima of the reaction: The O. D. value of the reaction mexture was measured at different wave lengths starting from 400–800 nm. The values were taken with the help of spectrocolorimeter (C. Z. instrument) and plotted on graph paper and the absorption maxima was derermined. Maximum absorption was at 620 nm.

Determination of standard curve of reserpine solution: To each of 1 ml of reserpine solution from 1000 ppm to 100 ppm, 1 ml of concentrated sulphuric acid (H_2SO_4) was added and optical density for each of the reaction mixture was determined with the help of spectrocolorimeter of 607 nm.

Isolation and purification of reserpine obtained from *R. serpentina* root by paper chromatography method: 500 mg of the root of *R. serpentina* was crushed into powder and was subjected to chloroform extract under reflux condition. The extraction was continued for 20 minutes and then filtered. The chloroform was evaporated to 0.5 ml of the extract. The crude extract was taken in a pipette and was subjected to paper (Whatman N 1) in a streak. The paper was run first in petroleum ether and then in pure methanol to obtain pure reserpine on chromatographic paper and its scheduled Rf. The paper was dried and then reserpine zone on the paper was cut into pieces. The pieces of paper were extracted with boiling chloroform and was filtered. The chloroform extract was evaporated to 0.5 ml. Acetic anhydride was added to make the total volume of 1 ml. 1 ml concentrated sulphuric acid (H_2SO_4) was added and O. D. value was determined at 807 nm. The percentage of reserpine was determined by dry weight basis.

RESULT AND DISCUSSION

The dried root of *Rauvolfia serpentina* has been used in indegenous system in medicine from ancient times in India. However its importance in modern medicine was recognised in 1952 only after the isolation of a pharmacodynamic

principle, reserpine in the roots of the plant which revolutionized the therapeutic use of the drug as antihypertensive and sedative. Since then a number of alkaloids have been isolated from the roots of the plant. the synthesis of reserpine in 1956 followed as a natural corollary to this necessity. But even after the marketing of the synthetic reserpine the natural product hold its place due to the lower price.

As reserpine is a secondary product its productivity is expected to vary in plants subjected to various treatments and conditions. Thus during investigation the quantitative estimation of reserpine in a large number of samples is very much needed.

Numerous methods have been reported for the quantitative estimation of reserpine after using UV spectrophotometry (Nguyen *et al.*, 1989), HPLC (Cieri, 1983), LC (Cieri *et al.*, 1987) fluorescence analysis (Balon-Almda *et al.*, 1986) and radio immunoassay (Arens *et al.*, 1978). But all these methods are cumbersome and sometimes involve costly instruments not available in all the laboratories. No colorimetric method has so far been established for quantitative estimation of reserpine. Thus an attempt has been made to work out an easy and rapid colorimetric method for the quantitative estimation of the natural product in pure condition.

Previously Siddiqui and Siddiqui (1931, 1935) isolated reserpine following the method of fractional separation and for obvious reason the fraction contained a mixture of alkaloids. The modern method of chromatography was not used during isolation of reserpine. It has been observed that during quantitative estimation of reserpine most of the authors did not apply chromatography for its purification. Here in this part of the work column chromatography and paper chromatography have been applied for isolation of pure reserpine on large and microscale respectively. During column chromatography a glass column was packed with alumina (Al_2O_3) in wet condition in petroleum ether. The chloroform extract of root of *R. serpentina* was concentrated and adsorbed on alumina. Different solvent

and their mixtures used during column chromatography have been shown in Table 2. Crystals of reserpine were obtained after elution with benzene : chloroform, 3:1 (Table 2). The pure reserpine was isolated very easily after recrystallisation from chloroform-methanol mixture. The isolated product showed m.p. 262°C similar to that of authentic reserpine. Moreover, the isolated product was confirmed to be reserpine after comparing the IR spectra of both the isolated product and reserpine. The absorption peaks (λ max) at 3370 (NH), 1690 (C=O), 1600, 1570 (aromatic C=C), 1440 (CH_2), 1400, 1360, 1320 (C–N) 1260, 1230, 1210, 1100 (C–O), 1050, 1020, 990, 790, 760 (o-substituted benzene + indole aromatic ring) and 730 cm^{-1} of the isolated product were observed to be similar to those of authentic reserpine (Fig. 2).

Table 2 : Purification of crude extract of root bark of *R. serpentina* by column chromatography.

Solvent and their mixtures	**Ratio**	**Fraction number**	**Residue obtained after evaporation**
Petroleum ether	Pure	1 – 5	Oil
Petroleum ether:Benzene	3:1	6 – 10	Oil
Petroleum ether: Benzene	1:1	11 – 15	Oil
Petroleum ether: Benzene	1:3	16 – 20	No residue
Benzene	Pure	21 – 25	No residue
Benzene. : Chloroform	3:1	26 – 30	Crystal
Benzene. : Chloroform	1:1	31 – 35	No crystal(residue)
Benzene. : Chloroform	1:3	36 – 40	No residue
Chloroform	Pure	41 – 45	No residue
Methanol	Pure	46 – 50	No residue

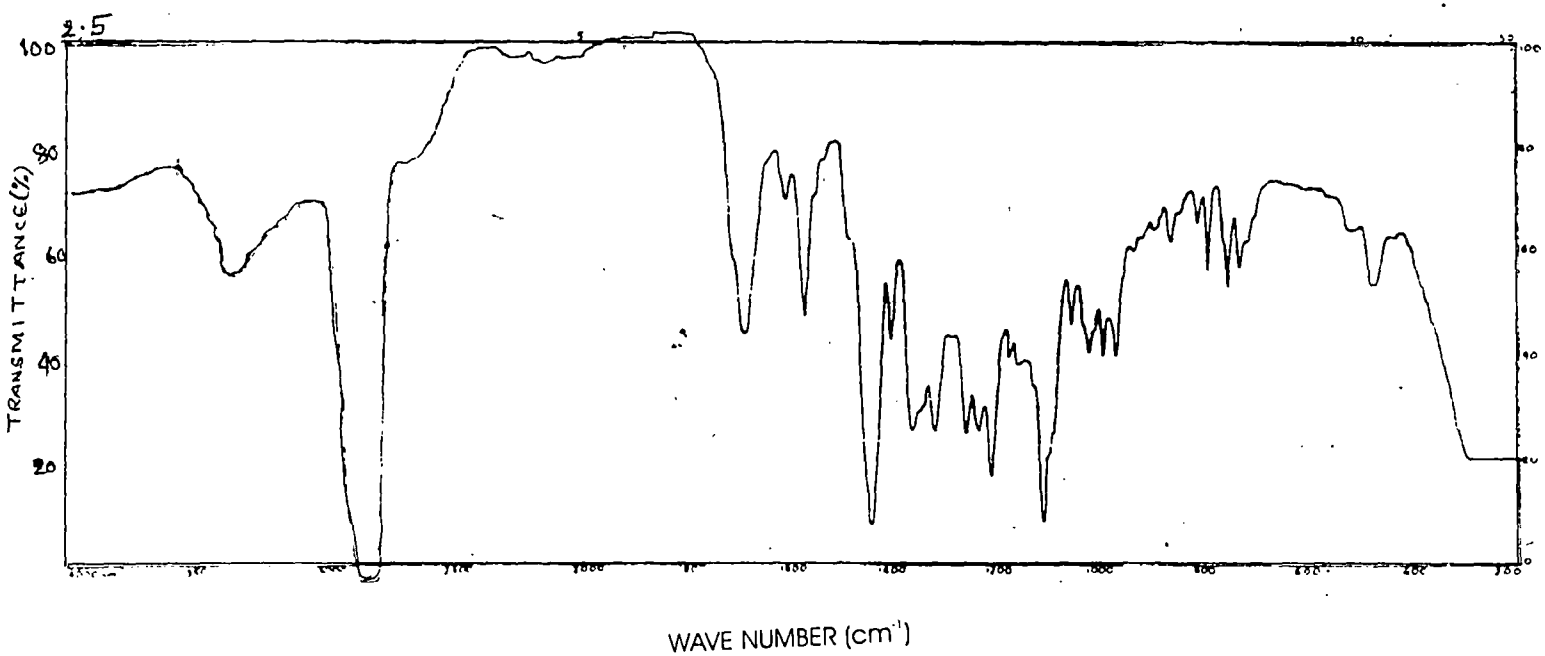


Fig. 2 IR spectrum of reserpine

Table 3 shows the Rf values of the natural product isolated from root bark of *R. serpentina* and which are similar to those of authentic reserpine in the same solvent or solvent mixtures during paper chromatography. These informations will be of much help during purification of extract containing reserpine on microscale by paper chromatography. In order to isolate pure reserpine the paper bearing reserpine was dried and the zone of paper bearing natural product corresponding to Rf of authentic reserpine was taken out and cut into pieces. These were subjected to hot chloroform treatment, filtered and chloroform was evaporated to obtain solid mass of reserpine which was used for its quantitative estimation with the help of new method proposed in this part of work.

The methodology was based on the principle that the reaction mixture containing reserpine, acetic anhydride and conc. H_2SO_4 shows uniform brown colour.

The colour complex was observed to show absorption maxima at 620 nm (Fig. 3) and the colour became stable after standing the mixture, for 15 to 20 minutes and continued to last for one hour. The reserpine content was calculated from the prepared standard curve ranging from 100 ppm to 1000 ppm and which was observed to obey Beer's Law (Fig. 4).

The proposed method is supposed to be advantageous because of the fact that reserpine content can be determined from a low concentration of 100 ppm solution. Moreover with this method only a small amount of dried plant issue was observed to be sufficient for estimation of reserpine content within a very short time.

The proposed method may be treated as first time to report in the field of quantitative estimation involved in pharmacognosy. It is claimed to be an easy method because it is very easy to separate reserpine from the crude extract by paper chromatography and the determination could be done with the help of colorimeter which is easily available in all the laboratories.

Table 3 : Rf value of authentic reserpine and unknown sample isolated from root bark of *R. serpentna* during paper chromatography in different solvents/solvent mixtures.

Reserpine/unknown compound	**Solvent used**	**Ratio v/v**	**Rf**
Reserpine	Petroleum ether	Pure	0.00
Reserpine	Petroleum ether:EtOH	9 : 1	0.19
Reserpine	Petroleum ether:EtOH	1 : 1	0.92
Reserpine	Benzene	Pure	0.40
Reserpine	Chloroform.:MeOH	1 : 9	0.92
Reserpine	Methanol	Pure	0.58
Reserpine	EtOH	Pure	0.675
Unknown sample	Petroleum ether	Pure	0.00
Unknown sample	Petroleum ether:EtOH	9 : 1	0.19
Unknown sample	Petroleum ether:EtOH	1 : 1	0.92
Unknown sample	Benzene	Pure	0.45
Unknown sample	Chloroform:MeOH	1 : 9	0.90
Unknown sample	Methanol	Pure	0.58
Unknown sample	EtOH	Pure	0.68

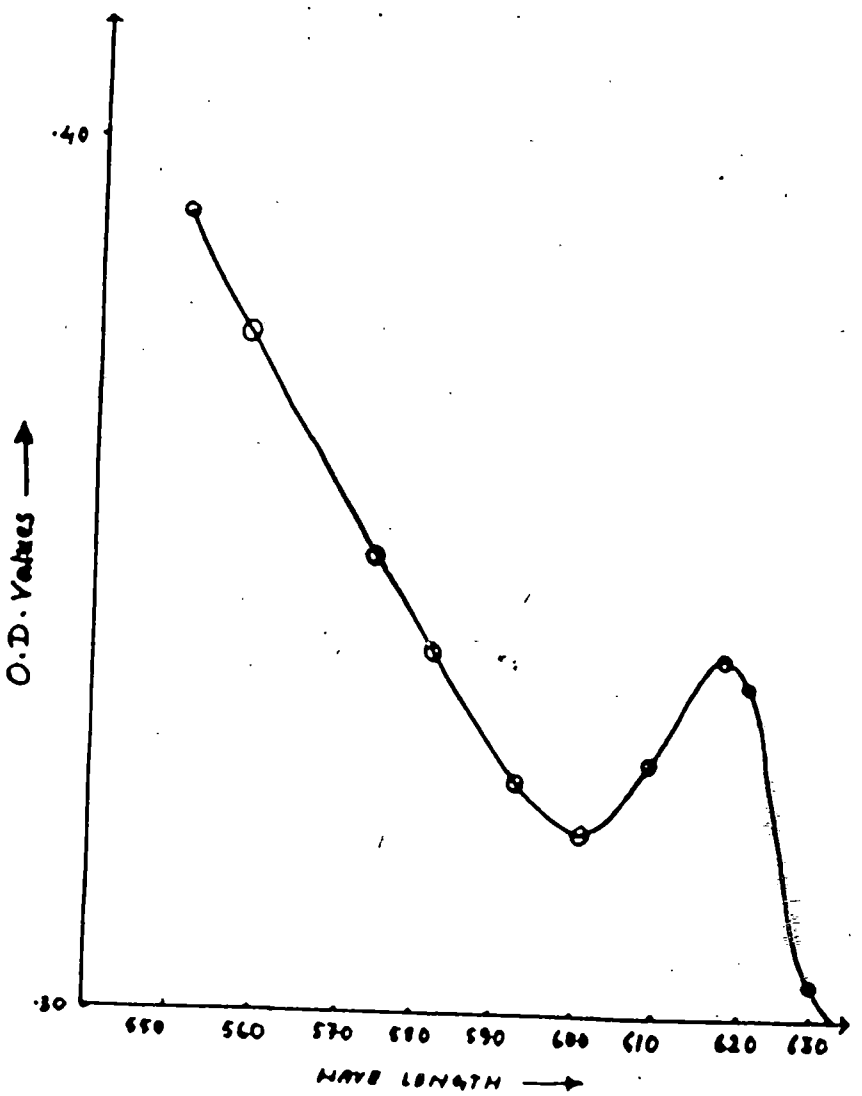


Fig. 3 Absorption maxima of reserpine

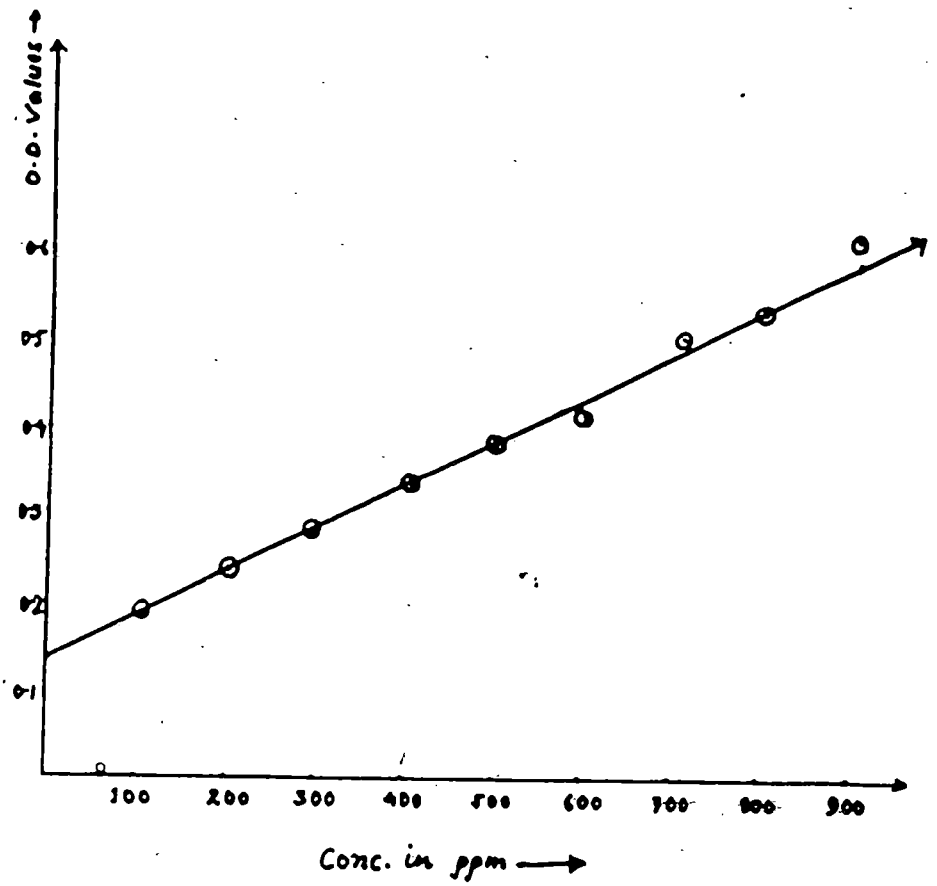


Fig. 4 Standard curve of reserpine

SUMMARY

Root barks of *Rauvolfia serpentina* were collected from different places of Darjeeling District. They were cut into pieces, sun dried and made powder. 500 g of powder was subjected to chloroform extraction with the help of soxhlet apparatus.

The chloroform extract after filtration was concentrated to small volume and subjected to column chromatography taking alumina as adsorbent. The column was eluted with different solvents such as petroleum ether, benzene, chloroform, methanol and their mixtures. Appeciable amount of crystals was obtained after eluting with benzene : chloroform (3:1) and recrystallised from chloroform-methanol mixture. The isolated product was identified to be reserpine having m.p. 262°C and after comparing absorption peaks in IR of isolated product with those of authentic reserpine.

Rfs of isolated product and reserpine in different solvent mixtures during paper chromatography were determined. For quantitative determination of reserpine, a reaction mixture was produced after mixing 1 ml of reserpine solution and 1 ml of concentrated sulphuric acid. It was shaken to produce uniform brown colour.

Absorption maxima of the reaction mixture was determined to be 620 nm.

Standard curve of reserpine from 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm was observed to obey Beer's law.

The brown colour of the reaction mixture attained stability after 15 to 20 minutes and continued to become stable for one hour.

CHAPTER 3

SEED GERMINATION AND CUTTING PROPAGATION OF *R. serpentina* IN ECOLOGICAL CONDITION OF DARJEELING DISTRICT

STUDIES ON SEED GERMINATION IN *R. serpentina*

INTRODUCTION

In the natural condition, *R. serpentina* is propagated by seeds. Owing to the hard sclerotic endocarp, the seeds require longer time to germinate as well as lower overall percentage of germination.

Santapau (1956) reported that germination of seeds of *R. serpentina* was poor and in general it was difficult to get more than 10% germination. Chandra (1956) found that more than 90% seeds of *R. serpentina* floated in water and among the heavy seeds, he reported a germination percentage of 43. Nayer (1956) also observed an irregular and sporadic germination of seeds and being 38% and 29% in case of Dehra Dun and South India lot, respectively. Badhwar *et al.* (1956a) advocated propagation of *R. serpentina* by seed and reported a germination percentage of 25 to 50.

Breaking of dormancy and improvement of germination percentages have been done with various physical and chemical treatments in a number of wild and cultivated plants (Sinha, 1977; Singh and Kumar, 1984; Singh *et al.*, 1993; Padma *et al.*, 1994; Raina *el al.*, 1994; Devi and Selvaraj, 1994).

To hasten germination, the most common treatment practised is mechanical scarification, which was originally developed by Hughes (1915). A

few chemicals have been used to eliminate dormancy and stimulate germination of seeds (Devi and Selvaraj, 1994).

The present investigation was taken to assess the effectiveness of different treatments for germination percentage of seeds of *R. serpentina*.

MATERIALS AND METHODS

Freshly collected seeds of *R. serpentina* were subjected to the following treatments :

(1) **Mechanical Scarification** : Individual seeds were rubbed against sand paper or grind stone or nicked with a needle.

(2) **Hot Water Soaking** : The seeds held in a netting wire were soaked in hot water at 80° ± 2°C for 5, 10, 15 and 20 minutes.

(3) **Sulphuric Acid Treatment** : The seeds were dipped in conc. sulphuric acid for 3, 5, 10, 15, 30, 45, 60 and 90 minutes, after which the seeds were thoroughly washed in running tap water and dried on paper towels.

(4) **Hydrochloric Acid Treatment** : As in sulphuric Acid Treatment.

(5) **Heat Treatment** : For dry heating, the seeds were exposed to temperatures of 70°, 80° and 90°C for 16, 24, 48, 72 and 96 hours duration in an oven.

(6) **Pre-sowing Seed Treatment with Chemicals** : Seeds were soaked for 24 hours in the following chemicals : 1% boric acid, 1% calcium hydroxide ($Ca(OH)_2$), 1% sodium dihydrogen phosphate (NaH_2PO_4), 1% potassium nitrate (KNO_3), 0.5% thiourea, 100 ppm GA_3 and 100 ppm NAA. Interactive effects of KNO_3 with GA_3 and NAA were also investigated.

Pre-soaked seeds were re-dried for 24 hours in a stream of air. Untreated seeds were used as control.

Germination tests were replicated three times and conducted in petri dishes of 9 cm diameter. Seeds were placed on two layers of blotting paper. A seed regarded as germinated when radicle was approximately 5 mm in length.

RESULTS

Among the various scarification treatments, seed scarification with sand paper was able to increase germination percentage to some extent (Table 4). On the other hand, grinding of seeds with stone or nicking with a needle were not effective in improving seed germination. The concentrated sulphuric acid and hydrochloric acid and hot water treatments could not prove to be effective in increasing germination percentage of seeds of *R. serpentina.*

Out of various treatments of the seed with conc. sulphuric acid, the treatments up to 30 minutes were able to increase germination percentage to some extent. Further increase in treatment period proved to be deleterious to seed germination. Similar results were obtained with conc. hydrochloric acid (Table 4).

Hot water treatment of *R. serpentina* seeds for different periods completely failed to improve seed germination.

Dry heating of the seeds for several hours also could not improve the germination percentage.

Primary seed treatment with different chemicals (Table 4) did not improve the germination percentage of *R. serpentina* seeds. Of the chemicals, potassium nitrate and thiourea appeared to improve germination of seeds to some extent. All other treatments were at par with each other and the control. As only one

concentration of those chemicals were used in the present investigation, further research is needed with more concentrations.

Table 4 : Effect of presowing seed treatments on % of germination of *R. serpentina*

Treatment	% of germination	Treatment	% of germination
Control	26	**Heat treatment**	
Scarification		70°C 16 h	28
Sand paper	48	24 h	28
Grind stone	32	48 h	32
Nicking	30	72 h	26
Hot water		96 h	28
5 min	26	80°C 16 h	28
10 min	24	24 h	30
15 min	28	48 h	32
20 min	28	72 h	30
Sulphuric acid		96 h	28
3 min	25	90°C 16 h	26
5 min	25	24 h	28
10 min	26	48 h	30
15 min	30	72 h	30
30 min	38	96 h	28
45 min	32	1% Boric acid	32
60 min	30	1% Calcium hydroxide	30
90 min	25	1% Sodium dihydrogen phosphate	30
		1.0% Potassium dihydrogen phosphate	30
Hydrochloric acid			
3 min	30	1.5% Potassium nitrate	40
5 min	28	0.5% Thiourea	30
10 min	32	100 ppm GA_3	32
15 min	26	100 ppm NAA	30
30 min	32		
45 min	28		
60 min	27		
90 min	27		

$LSD_{5\%}$ for comparing any two means = 7

In most of the treatments, seeds started germination at 12 days after sowing and continued upto 35 days. Speed of germination was not affected by the treatments.

DISCUSSION

Although *R. serpentina* can be propagated by both seeds and vegetative propagules, growth of the plants and root yield are better in those raised from seeds (Badhwar, 1956a). But germination of seeds is much lower (Dutta *et al.*, 1962; Nayar, 1956). Moreover, collection of seeds from wild sources is both laborious and costly, inasmuch as the plants grow sporadically and the seeds ripen a few at a time. If the ripe seeds are not collected in time, they drop off to the ground and are lost. For these reasons seeds are not easily available from wild sources. Therefore, in the present investigation attempts have been made to improve the germination percentage of seeds of *R. serpentina*.

As seed coat of *R. serpentina* is very hard, mechanical and chemical scarification methods were followed. Seed scarification with sand paper increased germination percentage to some extent, but grinding of seeds with stone or nicking with a needle were not effective. Sinha *et al.* (1993) also reported that scarification of seeds of *Trigonella corniculata* with sand paper was the most effective method for increase of germination. Singh *et al.* (1985) in lentil and Padma *et al.* (1994) in *Leucaena*, *Albizzia* and *Samanea* also observed similar result with sand paper. Contrary to the present results, Padma *et al.* (1994) reported increased percentage of germination with grind stone scarification and nicking.

In the present investigation, seed treatment with conc. sulphuric acid upto 30 min. increased germination percentage to some extent. This result corroborates the findings of Sinha *et al.* (1993), Padma *et al.* (1994) and Rao *et al.* (1985). However, the duration of soaking in sulphuric acid for better germination was different in different plant species.

The result of the present study revealed that hot water treatments of seeds for any duration and temperature did not improve germination percentage. These results are in agreement to the work of Jha and Sinha (1989) in *Vicia faba* and Sinha *et al.* (1993) in *Trigonella corniculata.* However, Padma *et al.* (1994) reported hot water (80°C) soaking for 5 minutes improved germination in *Leucaena leucocephala*, but not in *Albizzia lebbeck* and *Samanea saman.*

Presowing seed treatment with chemicals did not improve germination percentage. However, potassium nitrate and thiourea improve germination to some extent. Increased germination following treatment with potassium nitrate has been documented in several species like *Carica papaya* (Nagao & Furutani. 1986), *Citrus karna* (Singh *et al.*, 1979), *Glycine max* (Kalavathi, 1985) and *Momordica charantia* (Devi *et al.*, 1994). Increased germination following thiourea has been reported in marigold (Selvaraju, 1986) and bitter gourd (Devi *et al.*, 1994).

Basra *et al.* (1990) reported that seed soaking treatment with potassium nitrate, GA_3 and phthalimide increased seed germination in *Panicum maximum.* Devi *et al.* (1994) also observed that enhanced germination of bitter gourd due to seed soaking with a number of chemicals like bavistin, boric acid, calcium hydroxide, calcium oxychloride, sodium dihydrogen phosphate, potassium dihydrogen phosphate, succinic acid, NAA, cytozyme and mixtalol. But in the present study, some of these chemicals failed to elicit any positive effect on germination of *R. serpentina* seeds.

As mechanical and chemical scarification of seeds could not improve germination of *R. serpentina* seeds, it appears that germination inhibitors may be located inside the seeds. It is well known that presence of coumarins, phenolic and benzoic acid derivatives in seeds and plant parts inhibit their growth. Barton and Salt (1948) detected growth inhibitors in the aqueous extract of the seeds of *Sorbus* and *Berberis* and Siegel (1950) from the seeds of red kidney beans.

Sani and Datta (1969) examined air dry seeds of *R. serpentina* and *R. canescens* and found out that inhibition of germination may be ascribed largely to cinnamic acid derivatives and to a lesser extent to water soluble flavonoids, whereas in old seeds, the inhibition was largely due to hydroaromatic acids and to a lesser extent to cinnamic derivatives. The substituted coumarins, though present, appear to have negligible inhibitory effect.

The seeds of both the species of *Rauvolfia* accumulate more of hydrobenzoic acids on storage, probably synthesised from the breakdown processes of simple phenolics and aromatic compound 2,3- and 2, 5-dihydrobenzoic acids (0 – pyro catachuic and gentisic acids, respectively) in the old seeds of *R. serpentina*, inhibited lettuce seed germination markedly and inhibited root and shoot growth as well, to the same extent.

Out of the four substituted coumarins found in the extracts of fresh seeds of *R. serpentina* only imperatorin inhibited seed germination by 17%, but promoted root growth. Aesculetin, hernarin and umbelliferone had almost negligible effects on germination as well as on root growth.

Catechol was found in the fresh seeds of *R. canescens* but it had no inhibitory effect on germination.

Griffith (1958) has reported the presence of salicylic and genetisic acids in apocynaceous plants. But these two acids are not found in stored seeds of *R.*

serpentina and *R. canescens*. Probably there is a strong mechanism of converting these two acids as soon as they are formed so that the same remained absent in the seeds of *R. serpentina* or some different pathway is involved.

Torne (1964) subjected dry seeds of *R. serpentina* to irradiation with a series of gamma-ray doses ranging from 2,500 to 15,000 r. The irradiated seeds were immediately sown in pots along with untreated seeds as control. Results indicated that irradiation helped in increasing the percentage of germination from 20.5 in control to 74.5 and 73 in seeds with dosages 2,500 and 5,000 respectively. As the irradiation doses were increased further, germination percentage the gradually declined and it was 57.5% in seeds with 15,000 r dose.

From the above discussion it is clear that seed germination in *R. serpentina* is very complex and further work is necessary to understand the mechanism of germination.

DEVELOPMENT OF EASY PROPAGULES FOR CULTIVATION OF *R. serpentina* AND THEIR EFFECT ON ROOT PRODUCTION

INTRODUCTION

For obtaining roots for commercial purposes, cultivation of *R. serpentina* plants through seeds is the most economic method. But it is extremely difficult to obtain the required supply of seed from natural sources for raising commercial plantations immediately. Under the circumstances, there seems no other alternative than to undertake propagation by stumps, root cutting and stem cutting, which are more easily available. In fact, owing to the great demand for the drug, attempts are being made to raise *R. serpentina* plantation through vegetative propagation (Badhwar *et al.*, 1956b; Dutta *et al.*, 1963).

Moreover, germination of seeds of *R. serpentina* is very poor. In our experiment also, it was observed that germination percentage could not be increased substantially by physical and chemical scarification and with treatment with a number of chemicals.

Therefore, this study was undertaken to investigate the different methods of vegetative propagation of *R. serpentina* and the comparative performance of plants raised by different methods. Effect of different growth regulators on rooting behaviour of stem cuttings has also been studied.

ROOT FORMATION POTENTIAL OF DIFFERENT CUTTINGS OF *R. serpentina*

The aim of this experiment was to study the effect of stem, stump (stem-root junction) and root cuttings of *R. serpentina* on root formation.

MATERIALS AND METHODS

From 1.5–2 years' old plants of *R. serpentina* 15 cm long cuttings were prepared from three places i.e., stem, stump (stem-root junction) and root. These cuttings were used in the experiment. Cuttings were planted in polythene bags by inserting basal end (5 cm) in the soil.

Polythene bags of 10 cm diameter and 20 cm height were used. Polythene bags were filled with fine and ground soil mixed with cowdung (2:1) leaving 2 cm at the top. For drainage of excess water, each bag was pierced at several places at the bottom.

RESULTS

Results given in Table 5 shows that cuttings from stump revealed the highest root formation activity (78.57%) and it was followed by root cuttings

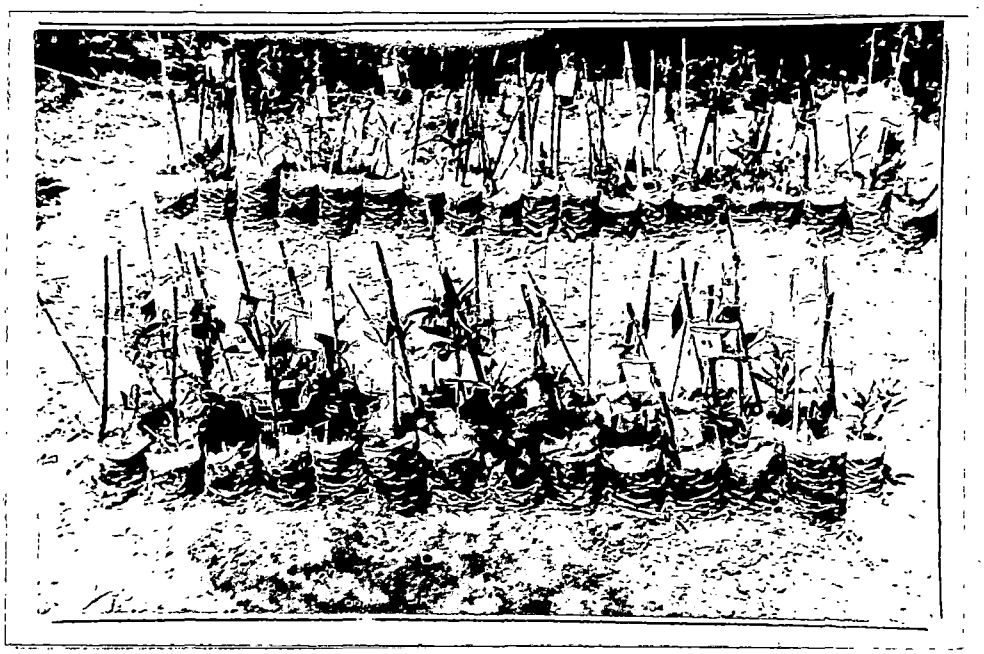
Table 5 : Root formation potential of different types of cuttings of *R. serpentina*.

Type of cutting	% of rooted cuttings
Stem	43
Stem-root junction	79
Root	63

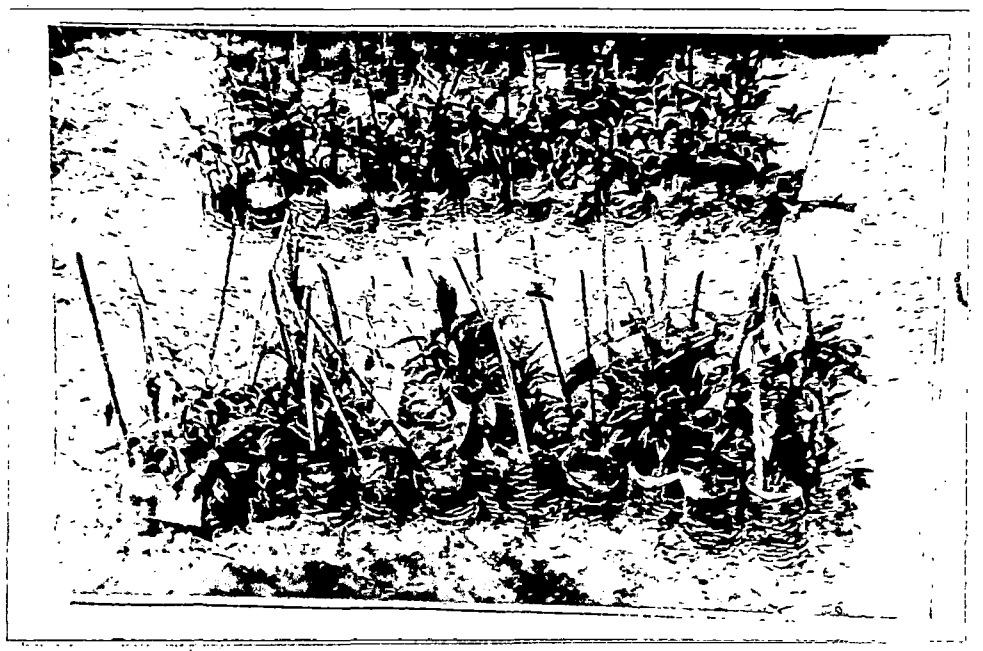
$LSD_{5\%}$ for comparing two means = 8

(62.82%) and stem cuttings (42.85%). This shows that, compared to others, stem cuttings had the lowest root formation activity. From stem cuttings, few plants

Photograph I. A view of *R. serpentina* in field condition



Photograph II. Propagules developed from stem cuttings.



Photograph III. Propagules developed from stem-root junction cuttings (upper) and root cuttings (lower).

were found to develop, whereas from others (stump and root cuttings), a good number of plants were found to develop.

EFFECT OF DIFFERENT GROWTH REGULATORS ON ROOTING BEHAVIOUR OF STEM CUTTINGS OF *R. serpentina.*

In the previous experiment, it was observed that stem cuttings of *R. serpentina* had the lowest root formation activity. Therefore, in this experiment, an attempt was made to improve root formation in stem cuttings of *R. serpentina* by applying different growth regulators.

MATERIALS AND METHODS

Stem cuttings (15 cm length) of *R. serpentina* were used for this purpose. Different growth regulators e.g., IBA, NAA and 2,4-D were used separately and IBA + NAA in combination in different concentrations (e.g., 1.5, 10, 50, 100, 200 and 500 ppm). Growth regulators were prepared in distilled water.

Initially the growth regulators were dissolved in ethanol or dilute NaOH, then the volume was made up to the mark with distilled water. For each treatment, equal number (6) of stem cuttings was taken and the basal part of the cuttings was dipped in the growth regulator solution till 48 hours, stem cuttings were transferred to prepared polythene bags (as in the previous experiment).

After treatment with growth regulator/ water, cuttings were inserted (5 cm) deep into the soil of polythene bags (2 cuttings/ bag). The polythene bags were kept out door under shade. Irrigation was done from time to time as per necessity.

RESULTS

Effect of IBA, NAA, their combinations and 2,4-D in different concentrations on the root formation in stem cuttings of *R. serpentina* is presented in Table 6. It is evident from the result that IBA at low concentration stimulated root formation. The highest stimulation (83%) was noted at 50 ppm IBA. With the

Table 6 : Effect of different growth regulators on root formation in stem cuttings of *R. serpentina*.

Growth regulators	Concentration (ppm)	Percentage of rooting	Growth regulators	Concentration (ppm)	Percentage of rooting
Control	-	43	IBA+ NAA	5+5	50
IBA	10	50		25+25	50
	50	83		50+50	50
	100	67		250+250	33
	200	50	2,4-D	1	50
	500	17		5	100
NAA	10	67		50	33
	50	33		100	00
	100	17			
	500	17			

$LSD_{5\%}$ for comparing two means = 12

further increase of concentration (100, 200 ppm), root formation was found to decrease gradually and at 500 ppm an inhibitory effect, compared to control was noted. At this concentration, the percentage of root formation was 17%. Only at low concentration (10 ppm) NAA stimulated root formation (67%). At higher

concentrations (50, 100 and 500 ppm) NAA inhibited the rooting activity of the cuttings. When used individually, both IBA and NAA at low concentration stimulated rooting activity. However, when they were used in combination and at low concentration, no synergistic effect was observed. IBA and NAA (5+5, 25+25, and 50+50 ppm) revealed 50% root formation activity. Only at more higher concentrations (250+250 ppm), IBA and NAA induced inhibitory effect. 2,4-D at very low concentration stimulated root formation on the stem cuttings. The highest activity (100%) was observed at 5 ppm of 2, 4-D. At 50 ppm it inhibited root formation and at 100 ppm it totally inhibited (100%) rooting activity of the stem cuttings.

DISCUSSION

Propagation of *R. serpentina*, particularly through seed is yet to some extent difficult. Cuttings from stem and other parts were tested for the development of propagules. Different growth regulators were also applied. All these growth regulators (IBA, NAA and 2,4-D) at low concentration increased root formation activity of the stem cuttings. But different combinations of IBA and NAA did not produce any synergistic effect on root formation activity of the cuttings, rather inhibitory effect was noted at high concentration. Chandra (1956) used different concentrations of IAA and NAA solutions on stem cuttings (12–15 cm long and 0.4–1 cm diameter) of *R. canescens* and did not observe stimulatory effect of these growth regulators on propagule development from those stem cuttings. Besides, cutting from stem-root junction without any growth regulators pretreatment may be effectively used for propagules development. In the present work, it was observed that stem cuttings developed 43%, stem-root junction cuttings developed 79% and root cuttings developed 63% of propagules of *R. serpentina* (Table 5). So, cuttings from stem-root junction may be considered very effective for development of propagules.

EFFECT OF TIME OF PLANTING AND TYPE OF CUTTING ON THE SUCCESS AND GROWTH OF *R. serpentina*

The aim of this experiment was to study the effect of time of planting and type of cutting on the success of cutting and subsequent growth of *R. serpentina.*

MATERIALS AND METHODS

From 1.5–2 years' old plants of *R. serpentina* 3–5 cm long root cutting (C_1) and about 15 cm long stem cutting (C_2) were planted in the experimental field of North Bengal University at 5 dates, i.e., 15 March (T_1), 15 April (T_2), 15 May (T_3), 15 June(T_4) and 15 July (T_5), 1998. The experiment was laid out in a randomized complete block design with a factorial arrangement of 5 times of planting and 2 types of cuttings. There were three replications. The cuttings were spaced at 30 x 20 cm. Weeding and watering were done as and when necessary.

Percentage of successful cuttings were recorded and growth and yield characters were noted after 1.5 years of planting. Data were analysed statistically.

RESULTS AND DISCUSSION

Mean squares from analysis of variance for some characters of *R. serpentina* are presented in Table 7. The results revealed that the effects of both time of planting and type of cutting were significant for percentage of success, plant height, no. of roots/ plant, length of longest root and air-dried root weight. Interaction between time of planting and type of cutting was also significant for all the characters except no. of roots/ plant and length of largest root.

Table 7 : Mean squares from analysis of variance for some characters of *R. serpentina.*

Sources of variation	df	% of success	Plant height (cm)	No. roots/ plant	Length of longest root (cm)	Air-dried root wt./ plant (g)
Replication	3	42	10	2.9	4	12
Time of planting (T)	4	4415**	1255**	34**	270**	1120**
Type of cutting (C)	1	1673**	434**	29**	89**	520**
T x C	4	210**	67**	5	3	160**
Error	27	35	12	3.1	6	16

** indicates significant at 1% level.

Main effects of time of planting and type of cutting are given in Table 8 and their interaction in Table 9. The results indicate that there was variation regarding % of success of cutting due to difference in planting time. The % of success of cutting varied from 18.4 to 59.7, being highest in 15 June planting and lowest in 15 March. The second highest value of 42.4% was recorded in 15 May. There was no statistical difference among 15 March, 15 April and 15 July plantings. Badhwar *et al.* (1956b) reported that the best month for raising rooted cuttings of *R. serpentina* under Dehra Dun condition to be June. Hartmann and Kester (1968) also reported similar results and they mentioned that broad-leaved evergreens usually initiate root most readily if the cuttings were taken after a flush of growth has been completed and the wood was partially matured.

Table 8 : Mean effect of time of planting and type of cutting on some characters of *R. serpentina*.

Treatment	% of success	Plant height (cm)	No. of roots/ plant	Length of longest root (cm)	Air-dried root weight/ plant (g)
Time of planting					
T_1	18.4	19.5	6.0	14.1	14.4
T_2	21.5	25.5	6.1	18.5	21.7
T_3	42.4	37.1	9.6	22.9	23.4
T_4	59.7	40.4	11.2	29.8	30.0
T_5	26.7	29.0	8.2	20.8	18.3
LSD 5%	6.1	3.6	1.8	2.5	4.1
Type of cutting					
C_1	38.2	33.5	8.9	22.7	24.3
C_2	29.3	27.1	7.5	19.7	18.7
LSD 5%	4.3	2.5	1.3	1.8	2.9

There was significant effect due to types of cutting on the % of success. The highest % of success of 38.2% was achieved from root while it was lowest (29.3%) from stem cuttings (Table 7). % of success of *R. serpentina* due to types indicated the superiority of root cuttings over stem cuttings. This is in agreement with the findings of Dutta *et al.* (1963). Stem cuttings are best prepared from woody twigs, tender green twigs have not been successful (Badhwar *et al.*, 1956b).

It has been noticed that although stem cuttings started aprouting 3–4 days after planting, they actually produced root after 60–65 days, producing 4 to 5 thin

rootlets. Most of the root cuttings started sprouting about 20–25 days after planting which corroborates the statement of Dutta and Chopra (1963) and Badhwar *et al.* (1956b). Chandra (1954) through his trial opined that root cuttings rooted within 10–15 days, produced healthy roots and shoots and that root cuttings of 1.2 cm diameter gave the best results.

Table 9 : Interacting effect of time of planting and type of cutting on some characters of *R. serpentina.*

Treatment		% of success	Plant height (cm)	No. of roots/ plant	Length of longest root (cm)	Air-dried root weight/ plant (g)
T_1	C_1	22.4	24.7	6.4	14.8	16.2
	C_2	14.4	14.3	5.6	13.3	12.5
T_2	C_1	26.8	26.7	6.3	19.5	23.6
	C_2	16.3	24.2	5.9	17.4	19.7
T_3	C_1	46.5	41.1	10.0	25.0	27.4
	C_2	38.3	33.0	9.1	20.8	19.3
T_4	C_1	65.2	45.2	12.4	31.9	34.2
	C_2	54.1	35.5	9.9	27.7	25.8
T_5	C_1	30.1	29.6	9.2	23.3	20.3
	C_2	23.3	28.3	7.2	19.3	16.3
LSD 5%		2.7	1.6	0.8	1.1	1.8

The combined effect of both factors namely time of planting and type of cutting showed no synchronized trend of significance regarding % of success

(Table 9). The highest % of success was noticed in 15 June planting in combination with root cutting.

Plant height due to different times of planting was found to be significant. The highest plant height was noticed when the cuttings were planted on 15 June and the lowest in 15 March planting. The difference between 15 April and 15 July plantings was not significant. Higher growth of plants of 15 June planting may be due to proper maturity of the cuttings. Because in *R. serpentina* it was observed by Gupta (1956) in India that both over-matured and immatured cuttings did not root easily with subsequent slow growth of the cuttings corroborating the present findings.

The results revealed that types of cuttings had significant effect on plant height. Root cuttings had higher height than stem cuttings. This is in agreement with the findings of Nayar (1956). Analysis of variance indicated the significant effect of interaction between the time of planting and type of cutting. Root cuttings when planted on 15 June produced the highest plant height (41.1 cm) (Table 9), but it was lowest in cuttings from stem planted on 15 March (14.3 cm).

The variation in total number of roots/ plant due to different times of planting was significant. Maximum number of roots (11.2) was recorded in 15 June planting, which was statistically identical to that of 15 May planting. The minimum number of roots (6.0) was achieved from 15 March, statistically similar to that of the 15 April planting.

Different types of cuttings had significant influence on the production of root. As usual the root cuttings produced the highest numbers of roots than the stem cuttings. There was no significant interaction between types of cuttings and times of planting in respect of root number.

Analysis of variance showed that the highest root length was significant by different types of planting. Maximum root length (19.8 cm) was recorded from 15 June planting which was statistically different from the remaining planting times and the minimum (14.1 cm) by 15 March planting. Gupta (1956) reported that both over-matured and the immatured cuttings could not root easily with subsequent slow growth of cuttings.

The differences in the length of the longest roots as influenced by different types of cuttings were significant. The highest root length was obtained from root cuttings and the lowest from the stem cuttings (Table 8). The interaction between times of planting and types of cuttings was non-significant.

Significant difference in respect of air-dried root weight/ plant was recorded due to different times of planting. Planting on 15 June resulted in maximum root weight (30.0 g) which differed significantly from the rest of the treatments. 15 April, 15 May and 15 July plantings produced statistically identical and 15 March planting produced the lowest root yield (14.4 g). Like other characters, root cuttings produced significantly higher root weight than the stem cuttings.

Analysis of variance indicated significant interaction between planting time and type of cuttings. The maximum root yield was obtained from the treatment combination of root cuttings and 15 July planting, while the minimum by 15 March x stem cuttings.

The results of the present study indicate that though *R. serpentina* could be propagated by both stem and root cuttings, better growth and root yield were obtained when propagation was done by root cuttings. These results also revealed that better growth and root yield could be achieved if root cuttings are planted on 15 June.

SUMMARY

R. serpentina is propagated by seeds. Owing to the hard sclerotic endocarp, the seeds require longer time to germinate and the overall germination percentage is low. Experiment was undertaken to assess the effectiveness of different treatments for germination percentage of seeds of *R. serpentina*.

Seed scarification with sand paper was able to increase germination to some extent. But grinding of seeds with stone or nicking with a needle were not effective in germination. The concentrated sulphuric and nitric acids and hot water treatments could not prove to be effective in increasing germination.

Presowing seed treatment with chemicals did not improve germination. However, potassium nitrate and thiourea improved germination to some extent.

As mechanical and chemical scarification of seeds could not improve germination of *R. serpentina* seeds, it appears that germination inhibitors may be located inside the seeds.

Although cultivation of *R. serpentina* through seeds is the most economic method, but it is extremely difficult to obtain the required supply of seed from natural sources. Therefore, there seems no other alternative than to undertake propagation by stumps, root and stem cuttings, which are more easily available. Study was undertaken to investigate the different methods of vegetative propagation of *R. serpentina* and the effect of different growth regulators on rooting of stem cuttings.

Stump cuttings had the highest root formation activity and it was followed by root cuttings and stem cuttings.

IBA at low concentration stimulated root formation of stem cuttings. The highest stimulation was noted at 50 ppm IBA. With the further increase of

concentration, root formation was decreased gradually. NAA also stimulated root formation in stem cuttings at low concentration only. 2,4-D at very low concentration stimulated root formation on the stem cuttings. The highest activity (100%) was observed at 5 ppm of 2,4-D and at 100 ppm it totally inhibited rooting activity of the stem cutting.

Experiment was undertaken to study the effect of time of planting and type of cutting on the success of cutting and subsequent growth of *R. serpentina*.

Both type of planting and type of cutting had significant effect on percentage of success, plant height, no. of roots/ plant, length of longest root and air-dried root weight.

The percentage of success of cutting varied from 18.4 to 59.7, being highest in 15th June planting and lowest in 15th March. There was no statistical difference among 15th March, 15th April and 15th July plantings. The highest percentage of success of 38.2% was achieved from root while it was lowest (29.3%) from stem cuttings.

Planting on 15 June produced the highest plant weight, maximum number of roots and highest root weight/ plant. 15 March planting produced the lowest root yield. Like other characters, root cuttings produced significantly higher root weight than the stem cuttings. Better growth and root yield of *R. serpentina* could be achieved if root cuttings were planted on 15 June.

CHAPTER 4

EFFECT OF NPK FERTILIZERS ON GROWTH AND DEVELOPMENT OF *Rauvolfia serpentina* WITH SPECIAL INTEREST ON THE PRODUCTIVITY OF ALKALOID CONTENT IN THE ROOT

INTRODUCTION

R. serpentina plants usually grow in natural habitat, i.e., in wild condition almost as an undergowth in deciduous forests. The defoliation of deciduous trees taking place annually in the natural habitat of *R. serpentina* ensures the availability of nutrients. But under plantation condition, regular supply of nutrients must be maintained for good growth and higher yields of root and alkaloid.

There are few reports which define fertilizer requirements for *R. serpentina* under different soil and agroclimatic conditions. Responses of N, P and K to root and alkaloidal yields have been recorded (Saini and Mukherjee, 1970; Sahu, 1970a, b; Nandi and Chatterjee, 1975; Maheswari *et al.*, 1988). Nandi and Chatterjee (1975) reported that single application of P and K could not increase the total alkaloid contents as compared to untreated plants, whereas N fertilizers, both singly and in combination augmented the total alkaloid contents. Maheswari *et al.* (1988) also observed that root and alkaloid yields of *R. serpentina* increased with N and P by 45 and 60 kg/ ha, respectively. Maurya *et al.* (1999) observed the effect of nitrogen on growth and yield of root of *R. serpentina* in the ecological condition of Bihar, India.

Nitrogen is an important component of amino acids and amino acids are generally considered to be the initial products of N assimilation. The initial recipients

of N in cell cytoplasm are different types of ketoacids. This happens through reductive amination and then transamination reaction. Amino acids are considered to be the important precurasors for the systhesis of alkaloids. As higher plants are not capable of excreting organically bound N, they have to store the nitrogenous wastes in safe place in nontoxic form. Alkaloids are considered to belong such categories of nitrogenous compounds. NPK fertilizers at higher doses, by increasing their influx into the cell may enhance their incorporation into cell constituent and especially N into ketoacids. In this way, synthesis of amino acids may be increased in the cell which in turn may increase the synthesis of alkaloids.

Although some work has been done on the effect of fertilizers on the root yield of *R. serpentina*, less information is available regarding the physiological basis of this effect. Variation in the dry accumulation and root yield caused by fertilizers application may be related to factors such as relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), relative leaf growth rate (RLGR), specific leaf area (SLA) and leaf weight ratio (LWR) (Buttery, 1969; Osman *et al.*, 1977; Paul, 1990a; Saha and Paul, 1988; Islam *et al.*, 1988; Paul and Saha, 1989; Paul and Sarker, 1989; Murtaza and Paul, 1986).

Growth analysis is the technique in quantitative analysis of plant growth, such as RGR, NAR and LAR. In recent years, growth analysis techniques have been extensively used by botanist, crop scientist and agronomist for a better understanding of the physiological basis of yield variation of crop plants. It is essential to quantify the components of growth and the variation, caused by NPK application, may be utilized in crop improvement for understanding the physiological basis of yield differences of *R. serpentina*.

The aim of this study was to find out the effect of NPK fertilizers on growth and root and alkaloid yields of *R. serpentina*.

EFFECT OF DIFFERENT COMBINATIONS OF NPK FERTILIZERS ON GROWTH AND YIELD OF *R. serpentina*

MATERIALS AND METHODS

The experiment was laid out in a randomized complete block design with 3 replications at the research field of the North Bengal University campus. The soil of the experimental field was silt and loam having a pH 7.3, low in organic carbon (0.44%), total N (0.43%), available P (15 ppm) and K (82 ppm). The field capacity of the soil was 32%. Five levels of NPK fertilizers were randomly assigned in each replication. The following 5 levels of NPK fertilizers were used in the present study :

Treatment	N (kg/ha)	P (kg/ha)	K (kg/ha)
T_0	0	0	0
T_1	30	20	15
T_2	60	40	30
T_3	90	60	45
T_4	120	90	60

Unit plot size was 1.8 m x 3.0 m. One month old seedlings were transplanted in the field on 10 May, 1998. 30cm x 30 cm spacing was followed. Each plot was

surrounded by a drain of about 90 cm. Proper cultural practices particularly weeding, digging and other activities were done as and when necessary. N, P and K fertilizers were applied in the form of urea, triple superphosphate and muriate of potash, respectively. The whole amount of the fertilizers were applied at the time of preparation of the field.

Growth Analysis

For growth analysis, four harvests were taken at equal intervals. The first harvest was taken at 30 days after transplanting and the successive harvests were at equal intervals of 15 days. From each replication, two plants were selected from each treatment. At the time of harvest, plants were cut down just at the ground level and the tops were separated into leaf, petiole and stem. Before weighing, all plants were separately dried in an oven at 90°C for 24 h till they reached constant weight.

Leaf area was measured by the disc method. Several discs were cut from the leaves from each treatment. Discs were weighed after oven-drying and leaf area was calculated by using following formula :

$$\text{Area of leaf} = \frac{\text{Area of discs X weight of leaves}}{\text{Weight of discs}}$$

From the leaf area and dry-weight, the following growth attributes were computed between two harvests according to the classical technique of growth analysis (Radford, 1967) :

$$\text{Relative growth rate (RGR)} = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}$$

$$\text{Net assimilation rate (NAR)} = \frac{(W_2 - W_1)(\log_e LA_2 - \log_e LA_1)}{(LA_2 - LA_1)(t_2 - t_1)}$$

$$\text{Leaf area ratio} = \frac{(\log_e W_2 - \log_e W_1)(LA_2 - LA_1)}{(W_2 - W_1)(\log_e LA_2 - \log_e LA_1)}$$

$$\text{Relative leaf growth rate} = \frac{(\log_e LA_2 - \log_e LA_1)}{(t_2 - t_1)}$$

The following growth attributes were calculated separately for each harvest :

$$\text{Specific leaf area (SLA)} = \frac{\text{Leaf area}}{\text{Leaf dry weight}}$$

$$\text{Leaf weight ratio} = \frac{\text{Leaf dry weight}}{\text{Total plant dry weight}}$$

Where W_2 and W_1 are the total dry weights, LA_2 and LA_1 are the leaf areas on two occasions t_1 and t_2, the first and second harvests, respectively.

Chlorophyll Content

Four discs, each of 0.5072 cm^2 area, were taken from four different positions of the fully matured leaf and chlorophyll was extracted with 80% aqueous acetone using a mortar and pestle to grind the tissues. The suspension was decanted into centrifuge tubes and centrifuged for 3 minutes. The clear green solution was decanted from the colourless residue and made up to 10 ml with 80% acetone. The optical density of this solution was determined against 80% acetone using a spectrophotometer at 645 and 663 nm. The quantity of chlorophylls a and b were determined according to the formulae given by Mackinney (1941) and later used by Arnon (1949) as follows :

$$\text{Chlorophyll a} = 12.717\ A_2 - 2.584\ A_1 = \text{mg chl. 'a' per litre}$$

$$\text{Chlorophyll b} = 22.869A_1 - 4.670A_2 = \text{mg chl. 'b' per litre}$$

Where A_1 and A_2 are optical density (O.D.) at wave lengths of 645 and 663 nm, respectively. Amount of chlorophyll per unit leaf area was calculated in the following way :

$$\frac{\text{mg chl. a or b per litre} \times 10}{\text{Leaf area} \times 1000}$$

Root Yield and Alkaloid Content

After 18 months of transplanting, plants were dug out in November, 1999 and the roots were separated and washed in running tap water. The roots were air-dried to constant weight. Six randomly selected plants from each treatment and replication

were used for this purpose. Composite root samples were analysed for total alkaloid and reserpine contents as in Chapter 2 of this study. Data were analysed statistically.

RESULTS AND DISCUSSION

Leaf Area and Dry Weight

Leaf area/ plant and total above-ground dry weight/ plant of *R. serpentina* as affected by growth stage and NPK fertilizers are shown in Figure 5. Both leaf area and total dry-weight of all the treatments were greater than the control at all the four growth stages.

The effects of N, P and K levels on these two characters were not pronounced at the early stages of growth, but at the later stages both leaf area and dry-weight showed an increasing trend with increasing N, P and K levels. For leaf area, the differences between T_3 and T_4 treatments were nonsignificant at most of the growth stages.

Growth Attributes

Relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), relative leaf growth rate (RLGR), specific leaf area (SLA) and leaf weight ratio (LWR) as influenced by different combinations of NPK fertilizers and growth stages are shown in Figures 6A –6C. The analysis of variance indicated that the effect of different combinations of N, P and K on these growth attributes were significant at all the growth stages except RLGR at (45–60), SLA at 60 and 75 and LWR at 45

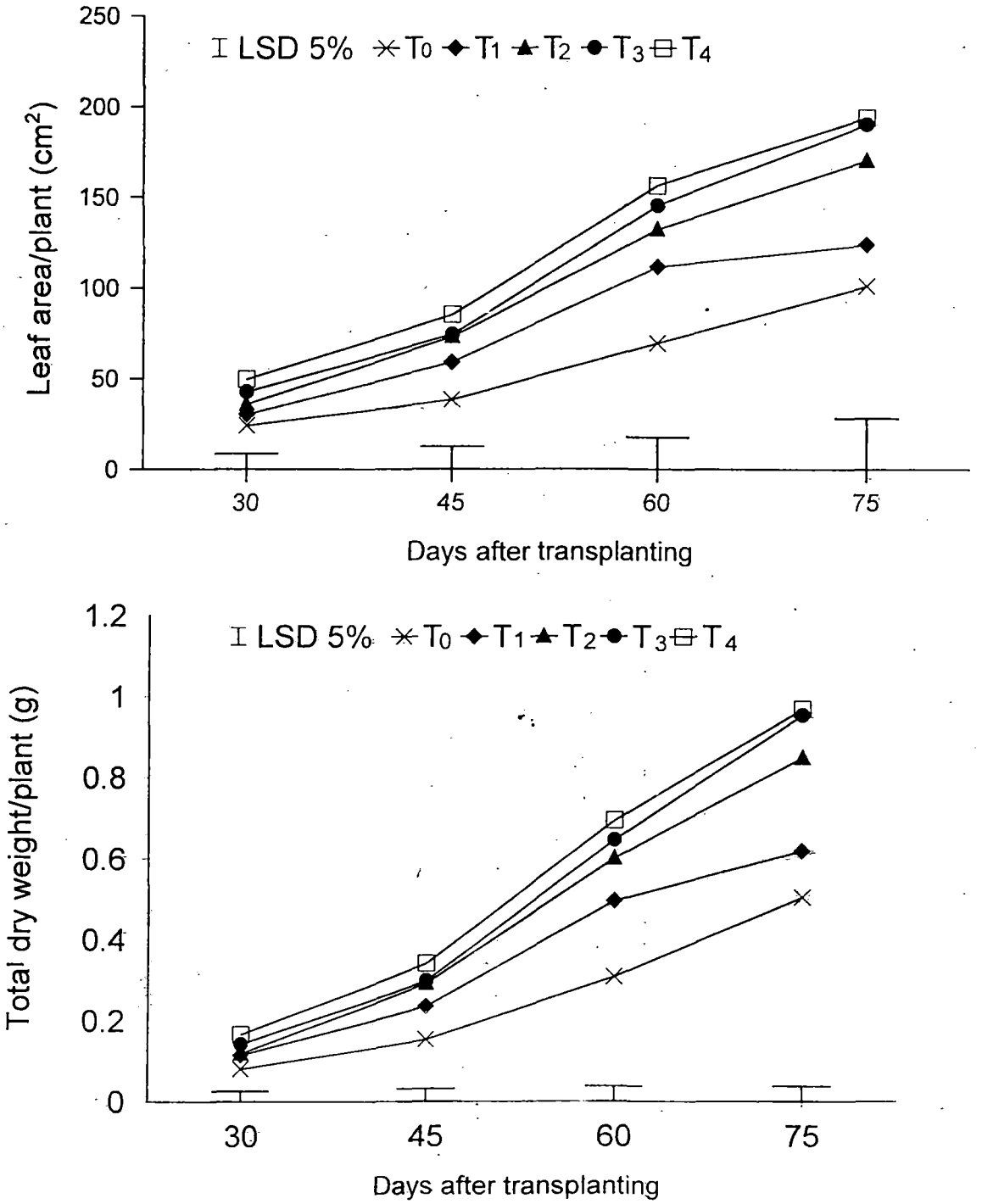


Fig: 5 Leaf area and total dry weight of *R. serpentina* as affected by growth stage and NPK fertilizers.

DAT. At (30–45) DAT, the highest RGR was observed in T_2, however, the difference between T_1 and T_2 was not significant. For T_0 (control) treatment, RGR gradually decreased with plant age, but for the remaining treatments RGR reached its peak at (45–60) DAT and then declined. Similar trend was observed for NAR, except T_0. Compared to T_0, T_1 and T_2, higher NAR was maintained in T_3 and T_4 at (60–75) DAT (Figure 6A).

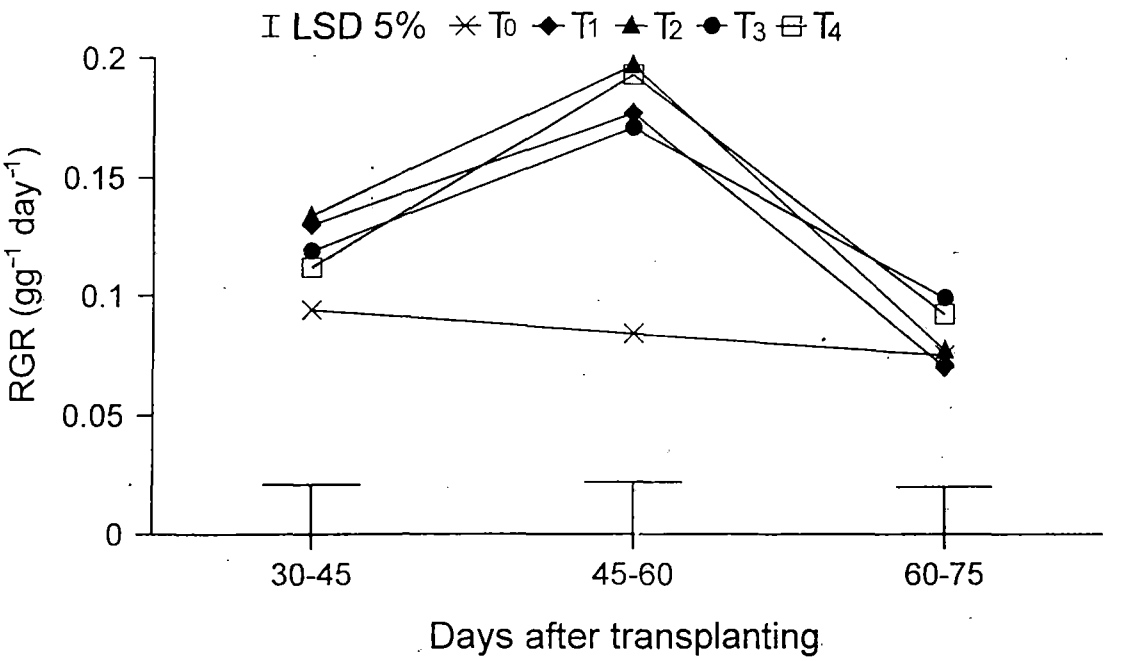
At the initial stage, significantly higher LAR was observed in T_0 and the differences between the remaining treatments were not significant (Figure 6B). LAR of all the treatments gradually decreased with plant age. At (60–75) DAT, significantly lowest LAR was obtained in T_4.

RLGR also decreased with plant age. At (30–45) DAT, T_2 showed the highest RLGR, which was not significantly different from T_1 and T_3 (Figure 6B). For the remaining two harvest intervals, there was no clear pattern of NPK fertilizer effect on RLGR.

SLA responded to NPK in different ways at different harvests (Figure 6C). At the first harvest, the highest SLA was observed at T_0 and the differences between the remaining treatments were not significant. The second harvest had the lowest SLA, increased at the third harvest and again decreased at the fourth harvest.

LWR, like SLA, responded in different ways at different harvests (Figure 6C). With the advancement of plant age, LWR decreased in all the harvests.

Earlier work on the nature of the effects of N, P and K on growth attributes is somewhat inclusive. El-Hattab *et al.*, (1980), Ramachandram and Ranga Rao (1980), Paul (1990a) and Saha and Paul (1988) reported that RGR and NAR were significantly affected by N application. On the other hand, Gopalakrishnan (1979),



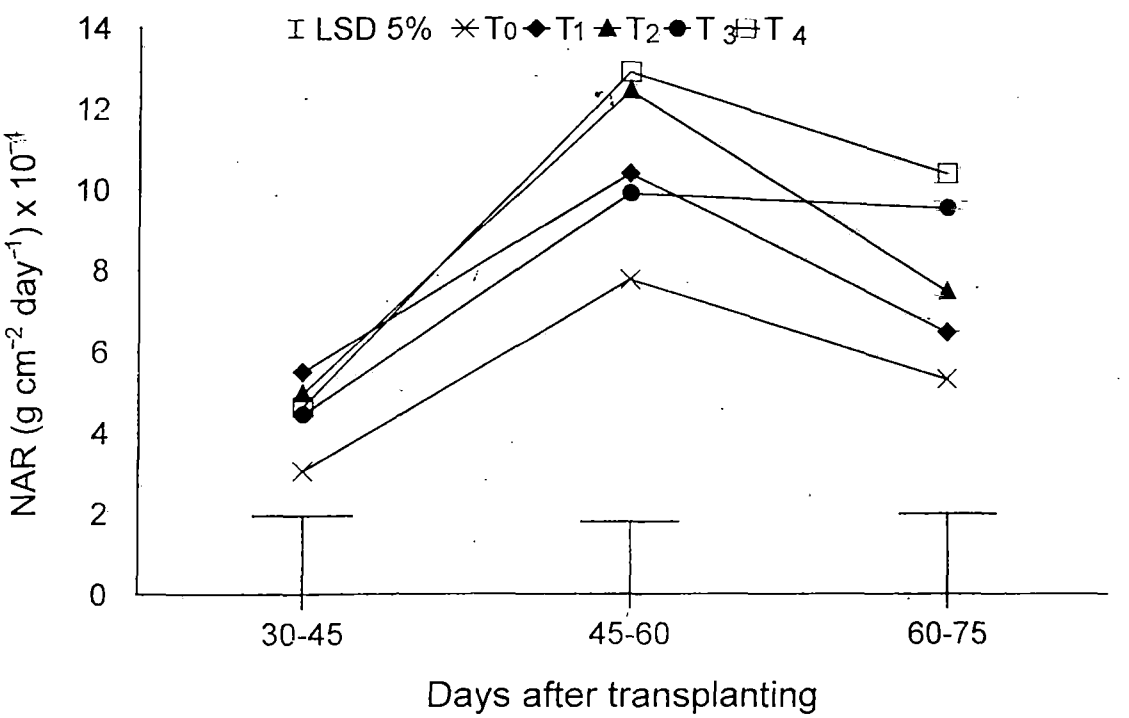


Fig: 6A RGR and NAR of *R. serpentina* as affected by growth stage and NPK fertilizers.

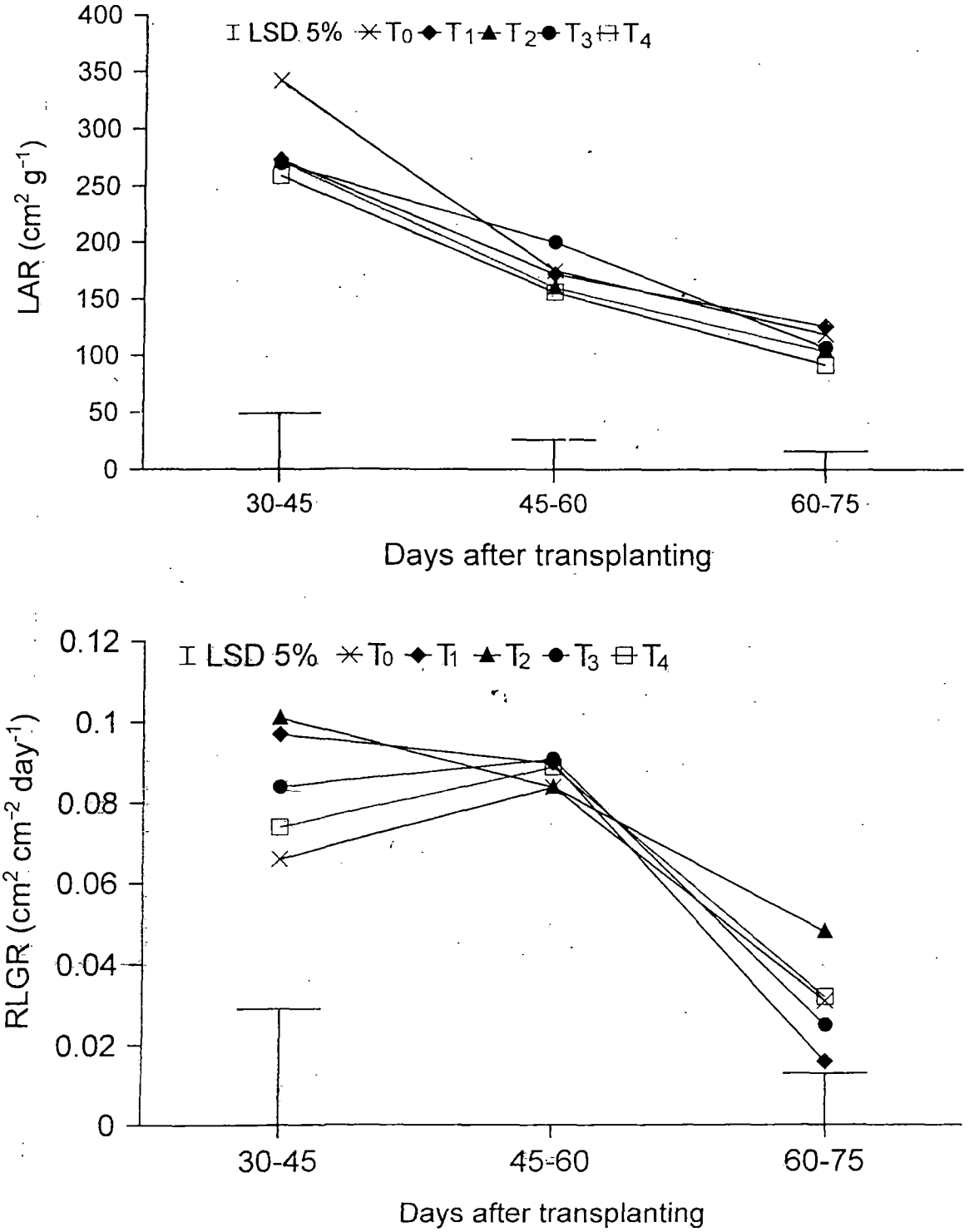


Fig: 6B LAR and RLGR of *R. serpentina* as affected by growth stage and NPK fertilizers.

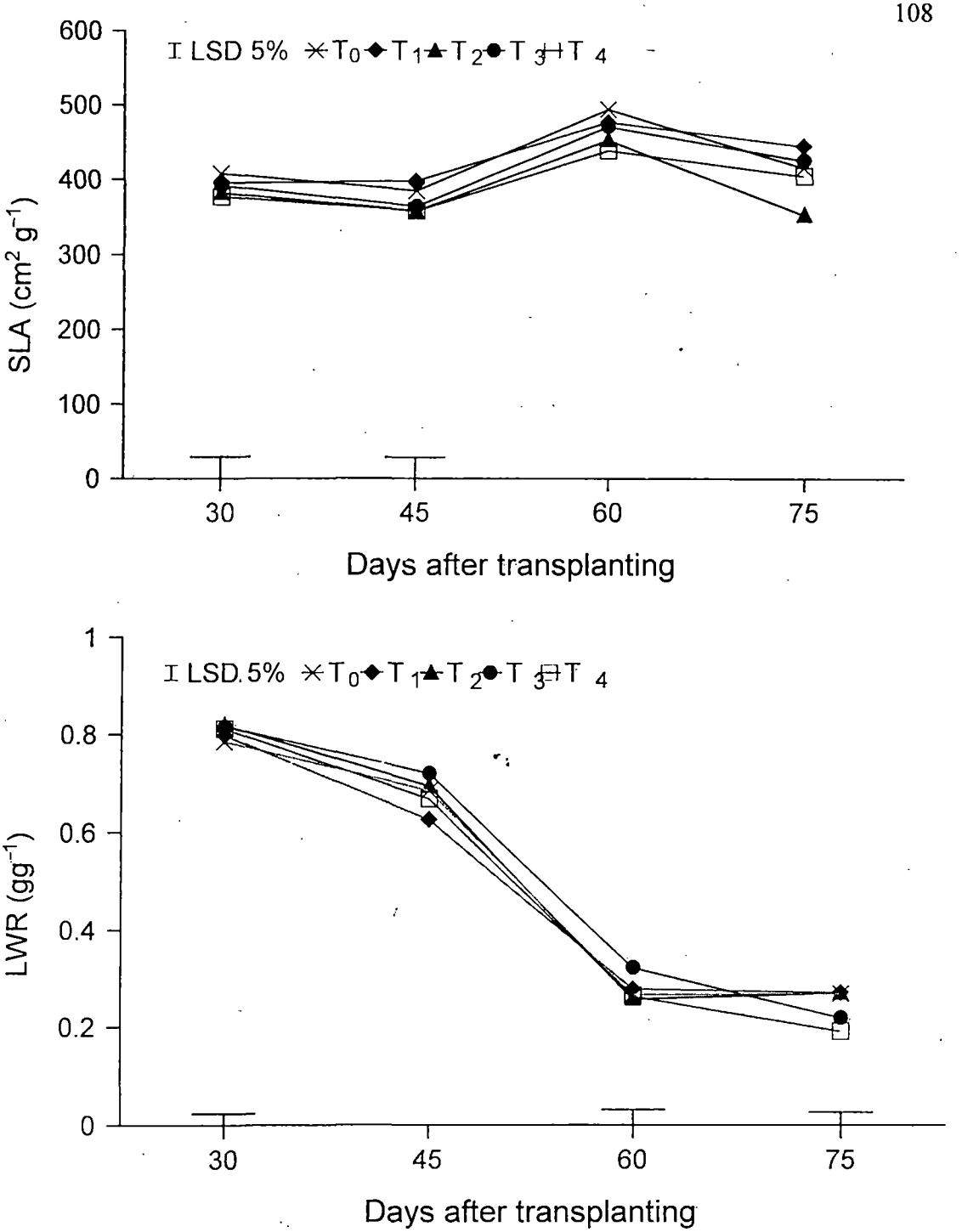


Fig: 6C SLA and LWR of *R. serpentina* as affected by growth stage and NPK fertilizers.

El-Shaer *et al.* (1979) and Murtaza and Paul (1986) did not find any significant effect N on RGR and NAR. Osman *et al.* (1977) observed that both RGR and NAR of wheat were unaffected by N application in the early stage of growth, but responded significantly to N in the time between 30 and 40 days from sowing. They further reported that the variation caused by the different levels of P and K was much less consistent. But Saha and Paul (1988) observed that compared to N and K, the different levels of P had pronounced effect on RGR and NAR. Islam *et al.* (1988) reported that at the initial stages, P and K alone and in combination produced higher RGR, but at the later stages, compared to N, RGR of P and K treated plants declined rapidly. They further reported that although at the initial stages, the effect of N on NAR was not marked, N maintained high NAR values at the later stages.

Paul (1990a) reported that RLGR was markedly affected by N application, but the effect of N was not so marked on LAR and its two components, SLA and LWR. Islam *et al.* (1988) observed that the effects of K and PK on LAR were much higher than N. El-Hattab *et al.* (1980) also observed that, in maize, LAR and LWR were decreased by increasing N rate and in ryegrass, Robson and Parsons (1978) noted that LAR was also decreased by increasing N rate at the initial growth stages, but this was reversed at the later stages of growth. In jute, Saha and Paul (1988) reported that the effect of different levels of NPK were significant for LAR and RLGR at most of the growth stages. But in rape seed, Murtaza and Paul (1986) were unable to find any effect of N on LAR and RLGR, but SLA and LWR were significantly affected by N levels.

Correlation Coefficients Between Growth Attributes and Root Yield.

Simple correlation coefficient between growth attributes themselves and with root yield are presented in Tables 10A, 10B and 10C respectively for (H_1–H_2), (H_2–H_3) and (H_3–H_4) harvest intervals. Leaf area had positive correlation with total dry weight and SLA at all the three harvest intervals and with LAR at (H_2–H_3) only. The positive association between total dry weight and RLGR was significant only at (H_1–H_2). RGR had positive correlation with LAR only at the initial growth stage, but it did not show any relationship with leaf area and dry weight at the initial and mid-growth stages, but showed positive association at the final stage. This indicated that the relationship between the growth attributes varied with plant age. The association between RGR and NAR was positive at the mid-stage. NAR was positively correlated with LAR at the initial stages. RGR was associated negatively with LWR at the mid and the final stages. LAR had positive association with both its components, SLA and LWR, at all the three growth stages. There was no relationship between SLA and LWR. Root yield had positive correlation with both RGR and NAR at all the three growth stages.

Since both NAR and LAR are the two components of RGR, it is important to examine the relative contribution of the assimilation (NAR) and the expansion of the photosynthetic surface (LAR) in determining dry matter production. The result of the present study indicated that changes of RGR was highly influenced by both NAR and LAR. The relative importance of these two components and their contribution to RGR have been reported previously by a number of workers. Similar to the present results, Paul and Shamsuddin (1988) and Shamsuddin and Paul (1994) observed that RGR of sweet potato had positive association with both NAR and LAR, but this

association changed with the harvest intervals. Islam and Paul (1986) observed that at the initial stages, NAR was responsible for variation of RGR of rape seed, but at the latter stages LAR was more important. However, Eagles (1967), Wilson and Cooper (1969), Thurling (1974) and Pandey and Sinha (1979) reported that NAR exerted more stronger influence on RGR than LAR.

Table 10A : Simple correlation coefficients between different growth attributes and root yield of *R. serpentina* at ($H_1 - H_2$) harvest.

	Total dry weight	RGR	NAR	LAR	RLGR	SLA	LWR	Root yield
Leaf area	0.876**	-0.018	-0.206	0.509	0.368	0.668**	0.215	0.325
Total dry weight		0.008	0.216	0.045	0.522*	0.278	-0.093	0.410
RGR			0.246	0.732**	0.589*	0.030	-0.165	0.685**
NAR				0.745**	0.617*	-0.487	-0.742**	0.780**
LAR					-0.165	0.927**	0.634*	0.235
RLGR						0.134	-0.405	0.125
SLA							0.380	0.225
LWR								-0.125

* and ** indicate significant at 5% and 1%, respectively.

Since LAR is the product of SLA and LWR, any changes in these components will certainly be reflected in the LAR. The results presented in Tables 10A, B and C

show that LAR exhibited highly significant and positive correlation with both SLA and LWR at all the harvest intervals. Similar results were also reported by Shamsuddin (1994) in Sweet potato, Islam and Paul (1986) in rape seed and Miah (1989) in mulberry. Highly significant positive correlation between LAR and SLA was reported by Hussain and Paul (1984) in jute. The results suggest that both the area of leaf displayed per unit of leaf weight (SLA), a measure of relative thickness, and the index of leafiness of the plant on the weight basis (LWR) played the important role in determining the rate of expansion of the photosynthetic surface in *R. serpentina*.

Table 10B: Simple correlation coefficients between different growth attributes and root yield of *R. serpentina* at (H_2 – H_3) harvest.

	Total dry weight	RGR	NAR	LAR	RLGR	SLA	LWR	Root yield
Leaf area	0.608*	0.051	-0.377	0.533*	-0.073	0.728**	-0.158	0.425
Total dry weight		-0.038	0.018	-0.220	-0.151	0.042	-0.585*	0.233
RGR			0.708**	-0.134	0.981**	-0.024	-0.592*	0.785**
NAR				0.746**	0.679**	-0.591*	-0.562*	0.733**
LAR					-0.125	0.899**	0.601*	0.505*
RLGR						-0.073	-0.106	0.115
SLA							0.271	-0.203
LWR								0.156

* and ** indicate significant at 5% and 1%, respectively.

Positive correlation of root yield with both RGR and NAR indicated that root yield could be increased by improving growth rate as well as the photosynthetic efficiency of the leaf.

Table 10C : Simple correlation coefficients between different growth attributes and root yield of *R. serpentina* at ($H_3 - H_4$) harvest.

	Total dry weight	RGR	NAR	LAR	RLGR	SLA	LWR	Root yield
Leaf area	0.793**	0.589*	0.006	0.348	0.303	0.639*	-0.162	0.125
Total dry weight		0.809**	0.234	-0.244	0.572*	0.105	-0.509	0.552*
RGR			0.173	-0.272	0.881**	0.186	-0.808**	0.545*
NAR				-0.213	0.573*	-0.288	-0.109	0.632*
LAR					0.344	0.863**	0.548*	-0.122
RLGR						0.073	-0.781**	-0.325
SLA							0.120	0.184
LWR								0.254

* and ** indicate significant at 5% and 1%, respectively.

Chlorophyll Content

Chlorophyll a content was significantly affected by NPK fertilizers (Table 11). Chlorophyll a was higher in all the treatments over the control (T_0) and gradually increased with the increase of NPK levels. There was no clear pattern of NPK fertilizers on chlorophyll b content. However, the total chlorophyll content gradually increased with NPK levels.

Table 11 : Chlorophyll content (mg dm^{-2}) of *R. serpentina* leaf as affected by NPK fertilizers.

Treatment	Chlorophyll a	Chlorophyll b	Total chlorophyll
T_0	1.86	0.80	2.66
T_1	2.15	0.65	2.80
T_2	2.42	0.85	3.27
T_3	2.62	0.78	3.40
T_4	3.18	0.86	4.04
LSD 5%	0.07	NS	0.17

Paul and Sarker (1989) reported that among the NPK treatments, chlorophyll content was higher in N and NPK. Islam *et al.* (1988) observed that chlorophyll content in rape was increased more by N fertilizer. Paul (1990b) also reported that in rape and turnip the chlorophyll content increased with the increasing N levels.

Root Yield and Alkaloid Content

Main root length, root yield and alkaloid content as affected by NPK fertilizers are given in Table 12. Analysis of variance indicated significant effect of NPK fertilizers on the main root length. Maximum length of main root was recorded in the highest level of NPK (T_4) which was statistically similar to that of T_3. Minimum length was observed in T_0 (control) and this was statistically at par with T_1.

Table 12 : Main root length, air-dried root weight and alkaloid content of roots of *R. serpentina* affected by NPK fertilizers.

Treat-ment	Main root length (cm)	Air-dried root weight/ plant (g)	% of increase over control	Total alkaloid (%)	% of increase over control	Reserpine (%)	% of increase over control
T_0	35	34	-	1.58	-	0.26	-
T_1	42	37	8.8	1.72	8.8	0.32	23.1
T_2	48	45	32.4	1.90	20.3	0.32	23.1
T_3	53	50	47.1	2.00	26.6	0.36	38.5
T_4	58	56	64.7	2.12	32.4	0.36	38.5
LSD 5%	8	12		0.32		0.05	

The different levels of NPK fertilizers showed significant variation on the air-dried root weight/ plant (Table 12). Root weight due to different levels of NPK

fertilizers varied from 34 to 56 g, being highest in T_4 (N_{120} P_{90} K_{60}) and lowest in T_0 (N_0 P_0 K_0). However, T_2 and T_3 were statistically at par with that of T_4.

NPK fertilizers had significant effect on total alkaloid and reserpine contents. Control plants produced the lowest amount in both cases. The remaining treatments increased alkaloid content slightly. Extra weight of roots obtained with NPK fertilizers, therefore, would result in proportionately greater absolute amounts of alkaloid content.

Little information is available regarding the effect of NPK fertilizers on root yield and alkaloid content of *R. serpentina*. Dutta *et al.* (1963) did not get any significant increase of root yield *R. serpentina* as a result of fertilizers application. Mukherjee (1970) observed that in *R. serpentina*, extra application of N resulted in an increase in reproductive growth but without any effect on root yield.

But Sahu (1970a) reported higher dried root yield of *R. serpentina* due to application of N and P fertilizers. Maheswari *et al.* (1988) also reported that N and P appreciably increased dried root yield of *R. serpentina*.

In the present investigation, NPK fertilizers increased total alkaloid and reserpine contents (Table 12). Nandi and Chatterjee (1975) reported that single application of P and K could not increase the total alkaloid contents as compared to untreated plants, whereas N fertilizer, both singly and in combination, increased alkaloid content. Maheswari *et al.* (1988) observed that total alkaloid contents in root of *R. serpentina* were increased by increasing level of both N and P fertilizers.

The overall result of the present experiment indicated that leaf area, dry matter and growth attributes, such as RGR, NAR and LAR were increased with increasing levels of NPK fertilizers. Root yield and alkaloid contents were also increased by NPK fertilizers. T_4 treatment ($N_{120}P_{90}K_{60}$) produced 64.7% more root yield and

32.2% more alkaloid than that of the control ($N_0P_0K_0$) (Table 12). Therefore, $N_{120}P_{90}K_{60}$ level of NPK fertilizers is recommended for higher root and alkaloid yields of *R. serpentina*. Further, correlation study indicated that root yield had positive association with RGR and NAR. It may be concluded that for selection of higher yielding cultivar of *R. serpentina*, emphasis should be given to higher RGR and NAR.

INFLUENCE OF DIFFERENT LEVELS OF N, P AND K ON GROWTH AND YIELD OF *R. serpentina*

The aim of this experiment was to study the effect of different levels of N, P and K on leaf area and dry matter production, growth attributes, chlorophyll content and root and alkaloid yields of *R. serpentina*.

MATERIALS AND METHODS

The experiment was conducted at the research field of North Bengal University campus. The experiment was arranged in a randomized block design with three replications. Each replication had 10 plots. In each plot, each of the following fertilizers treatments were applied : N_1 = 30 kg N/ha, N_2 = 60 kg N/ha, N_3 = 90 kg N/ha, P_1 = 20 kg P_2O_5/ha, P_2 = 40 kg P_2O_5/ha, P_3 = 60 kg P_2O_5/ha, K_1 = 15 kg K_2O/ha, K_2 = 30 kg K_2O/ha, K_3 = 45 kg K_2O/ha and C = Control (no fertilizer was added). All the fertilizers were added to the field one day before transplanting.

Healthy and uniform seedlings of 45 days old were transplanted in the field on 25 – 27 May, 1998. Necessary cultural practices were followed.

The plants were harvested 4 times : the first harvest was taken at 30 days after transplanting (DAT) and thereafter 15 days interval. At each harvest, plants were divided into leaves, petioles and stems and the plant parts were dried at 95°C for 24 h in an oven and then weighed. Leaf area was measured by the disc method. From dry weight and leaf area data, different growth parameters were calculated by following the classical growth analysis technique (Radford, 1967) as in the previous experiment. Chlorophyll content of the fully matured leaf was determined as in the previous experiment.

After 18 months of planting, plants were dug out in November, 1999 and the roots were separated and washed in the running tap water. Length of the main root was recorded. The roots were air-dried to a constant weight. Four randomly selected plants from each treatment and replication were used for this purpose. Composite root samples were analysed for total alkaloid and reserpine content as in Chapter 2 of this study. Data were analysed statistically.

RESULTS AND DISCUSSION

Leaf Area and Total Dry Weight

Changes in leaf area development and dry matter production of *R. serpentina* as influenced by different N, P and K levels are shown in Figures 7 and 8 respectively. Both leaf area and dry matter were gradually increased with time. The effects of N, P and K levels on leaf area and total dry weight were not pronounced at the early stages of growth, but at the later stages both the characters showed an

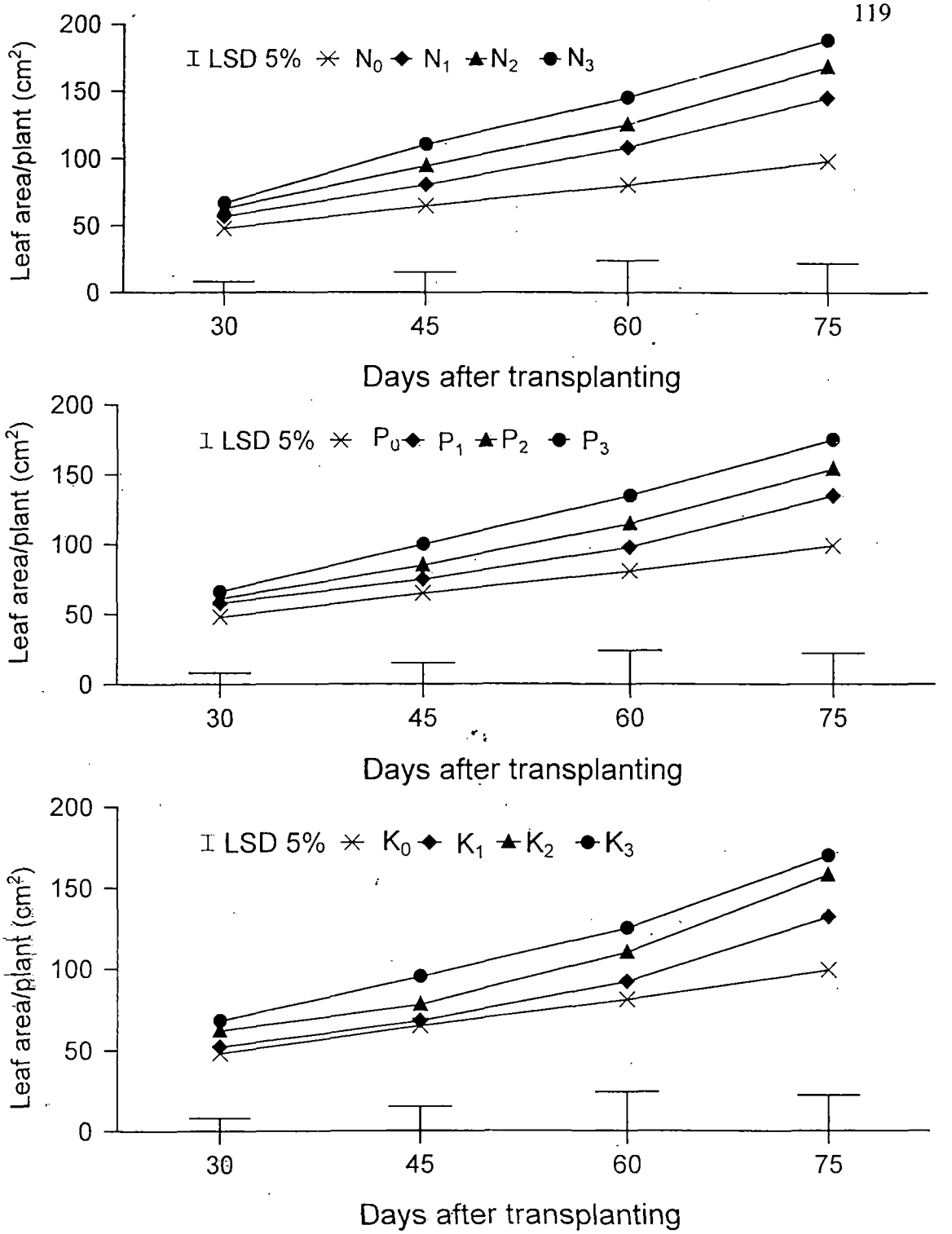


Fig: 7 Leaf area of *R. serpentina* with different levels of N, P and K at four harvests.

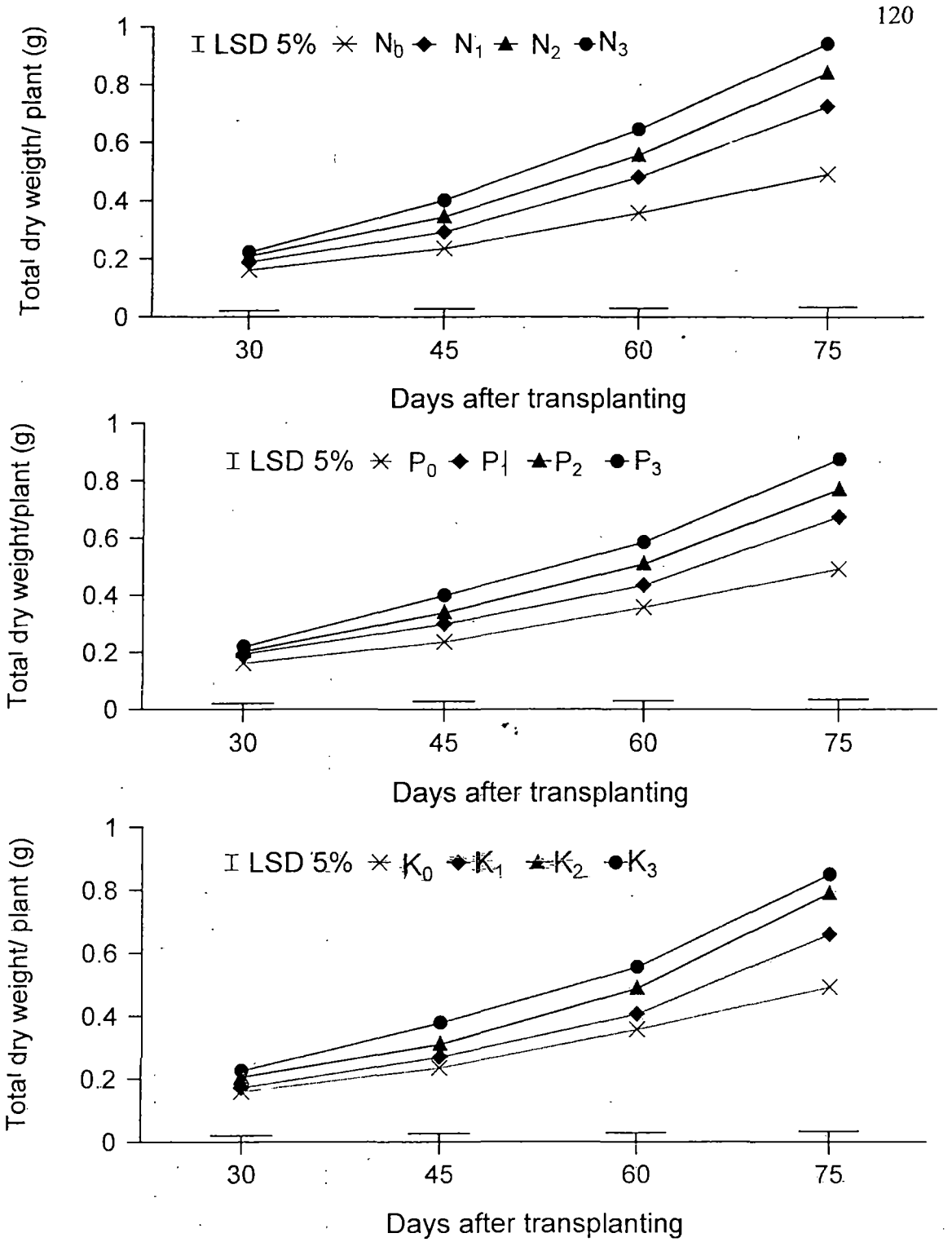


Fig: 8 Total dry weight of *R. serpentina* with different levels of N, P and K at four harvests.

increasing trend with increasing N, P and K levels. Osman *et al.* (1977) in wheat and Saha and Paul (1988) in jute reported similar results. Paul (1990a) also reported that higher levels of N delayed seedling emergence in rape and turnip and hence the leaf area and dry weight of high nitrogen-treated plants were lower at the initial stages of growth. The effect of N, within the range of application given in the experiment, was much larger than the K and P responses and this agrees with the findings of Osman and Milthorpe (1971) and Osman *et al.* (1977) in wheat, Saha and Paul (1988) in jute, Paul and Sarker (1989) and Paul and Saha (1989) in mustard. A contradictory result was reported by Buttery (1969) in soybeans where he indicated that fertilizers application significantly reduced the mean area per leaf, but increased the dry weight per unit area in the early stages of growth. Nandi and Chatterjee (1975) observed that high level of P only augmented the lamina area of *R. serpentina*, whereas N applied, both singly and in combination, decreased it.

Growth Attributes

RGR, NAR, LAR, RLGR, SLA and LWR as affected by N, P and K levels and growth stages are shown in Figures 9 –14. The analysis of variance indicated that effect of different levels of N, P and K on these growth attributes were significant at most of the growth stages. RGR, NAR and RLGR responded much by the application of N, P and K fertilizers. Compared to P and K, the different levels of N had pronounced effects on RGR, NAR and RLGR. Osman *et al.* (1977) reported that RGR of wheat was unaffected by N application in the early or late stages of growth, but responded significantly to N in the time between 30 and 44 days from sowing. Increases of RGR caused by increases in P and K levels were smaller than those caused by N increments, not particularly consistent and only marginal significance.

They further reported that like RGR, at the 30 and 37 days after sowing, NAR responded to increments of N, but the variation caused by the different levels of P and K was much less consistent.

RGR and NAR were markedly affected by N application in safflower (Ramachandram and Rango Rao, 1980), in maize (El-Hattab *et al.*, 1980), in jute (Saha and Paul, 1988) and in rape and turnip (Paul, 1990a). Saha and Paul (1988) also reported that compared to N and K the different levels of P had pronounced effect on RGR, NAR and RLGR. However, RGR and NAR were not significantly affected by N in cotton (El-Shaer *et al.*, 1979), in jute (Gopalakrishnan, 1979) and in rape (Murtaza and Paul, 1986).

With the increase of N, P and K levels, LAR gradually increased (Figure 11). However, LAR decreased with the advancement of plant age. Although the effect of different levels of N, P and K on SLA and LWR were less pronounced, these two growth attributes were also increased with increasing level of N, P and K. Earlier work on the nature of the effects of N, P and K on growth attributes is somewhat inconclusive. Robson and Parsons (1978) noted that LAR and SLA were decreased with increasing N rate at the initial growth stages, but this was reversed at the later stages of growth of ryegrass. El-Hattab *et al.* (1980) also observed that in maize, LAR and LWR were decreased due to increasing N rate. Saha and Paul (1988) observed that the effects of different levels of N, P and K were significant for LAR, SLA and LWR at most of the growth stages. Murtaza and Paul (1986) reported that LAR of rape was not significantly affected by N levels, but SLA and LWR were significantly affected. Paul (1990a) reported that the effect of N was not so marked on LAR and its two components, SLA and LWR, although the N effect was significant on LAR and LWR. Buttery (1969) also reported similar results in soybean.

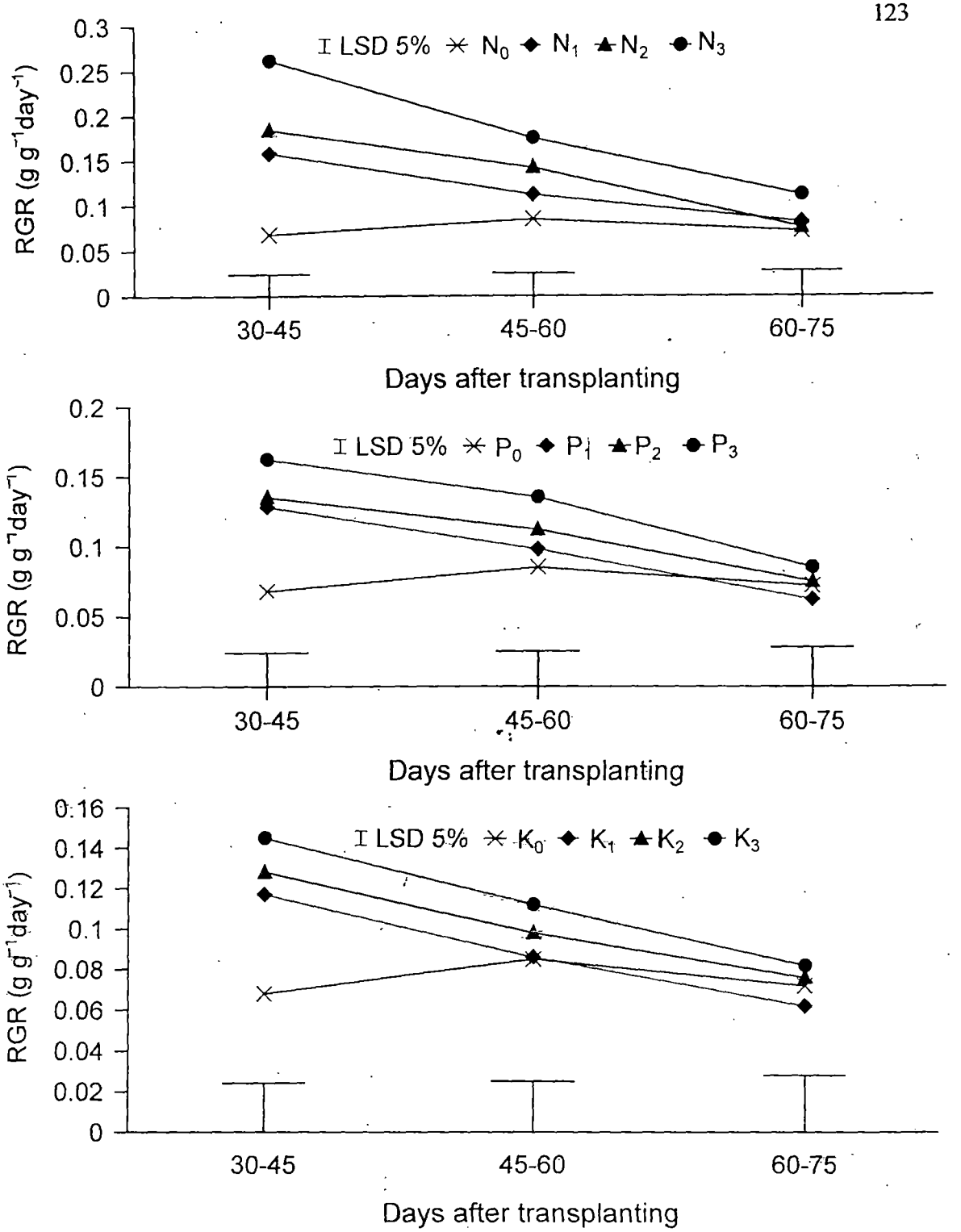


Fig: 9 Effect of different levels of N, P and K on RGR of *R. serpentina* at different stages of growth.

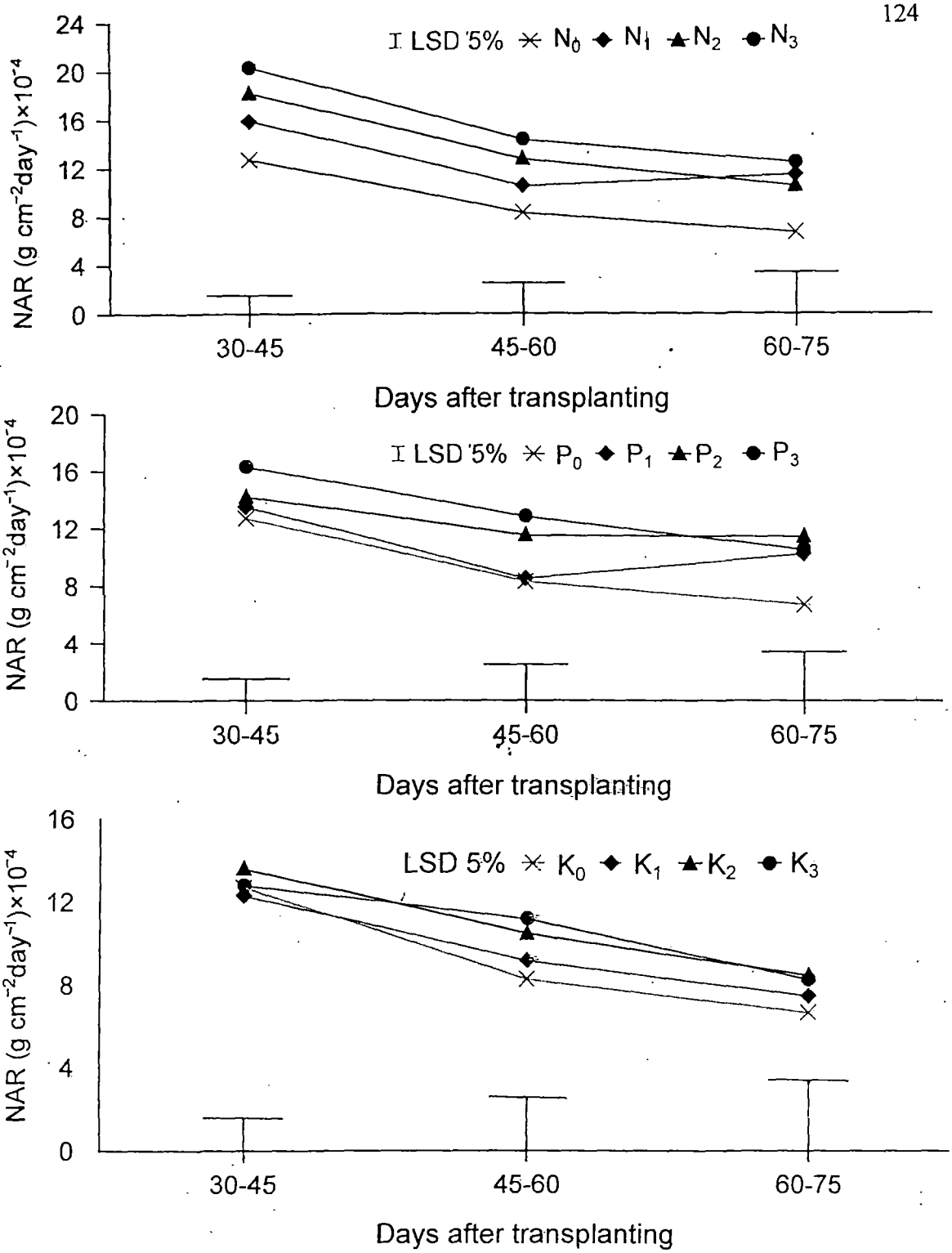


Fig: 10 Effect of different levels of N, Pand K on NAR of *R. serpentina* at different stages of growth.

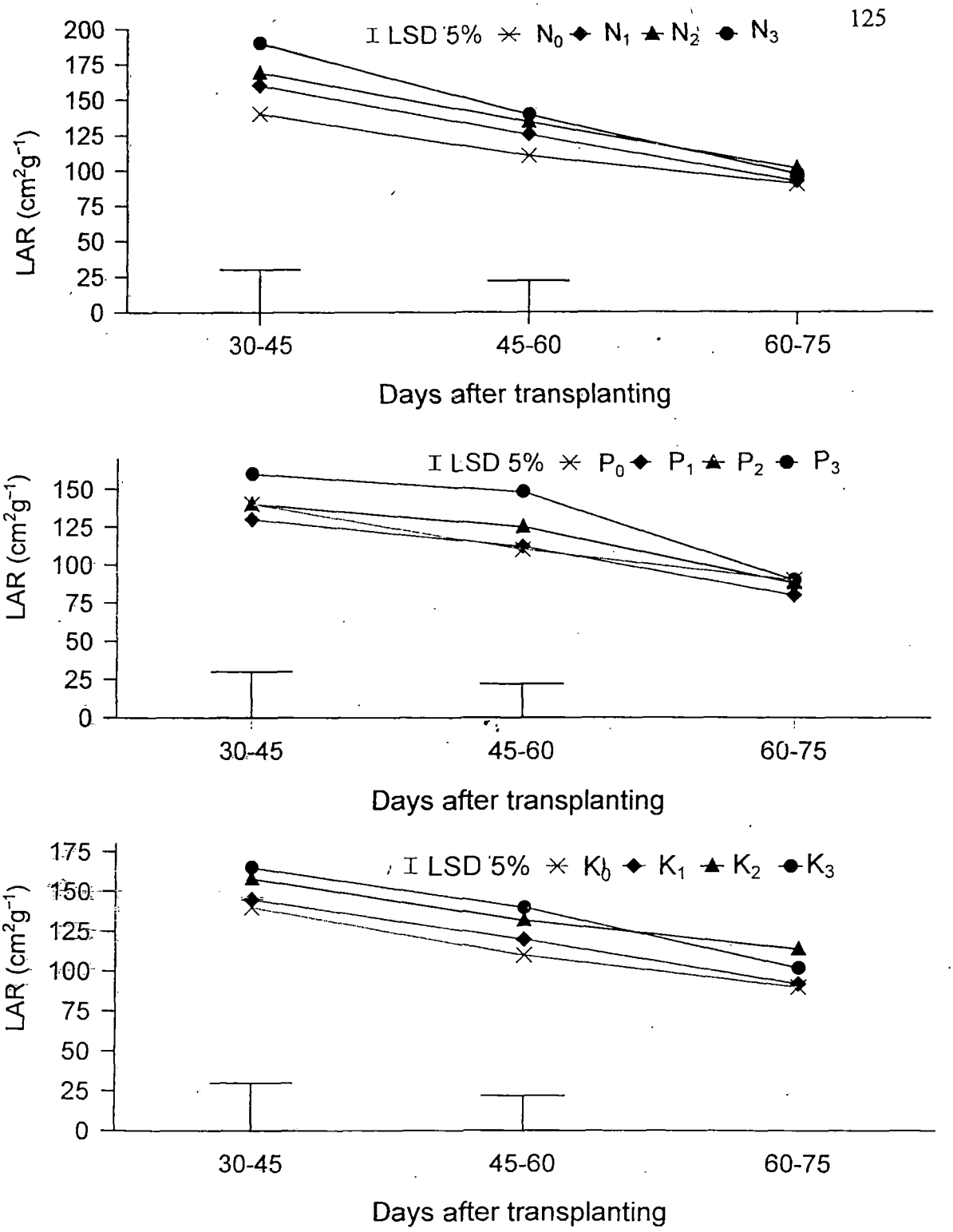


Fig: 11 . Effect of different levels of N, P and K on LAR of *R. serpentina* at different stages of growth.

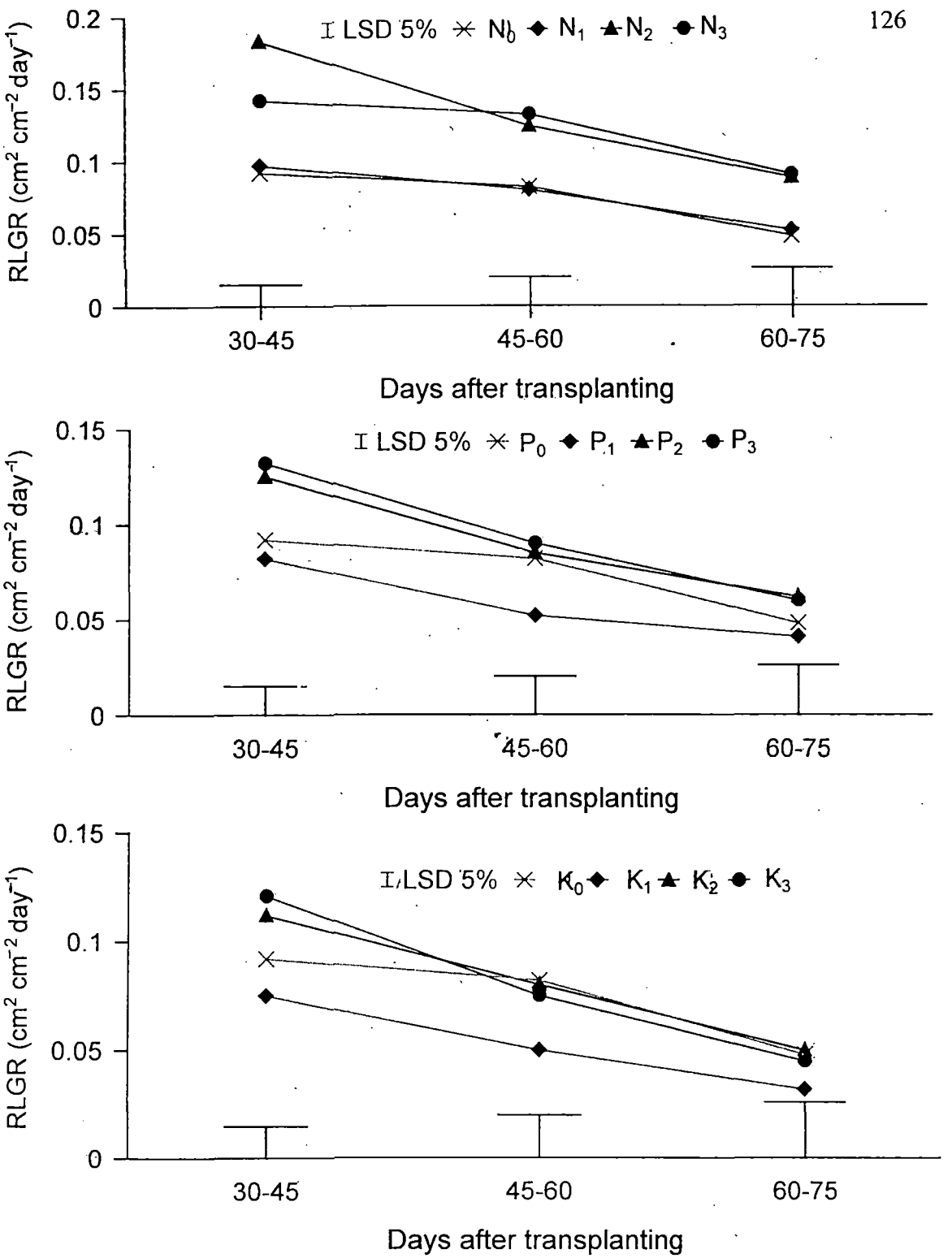


Fig: 12 Effect of different levels of N, P and K on RLGR of *R. serpentina* at different stages of growth.

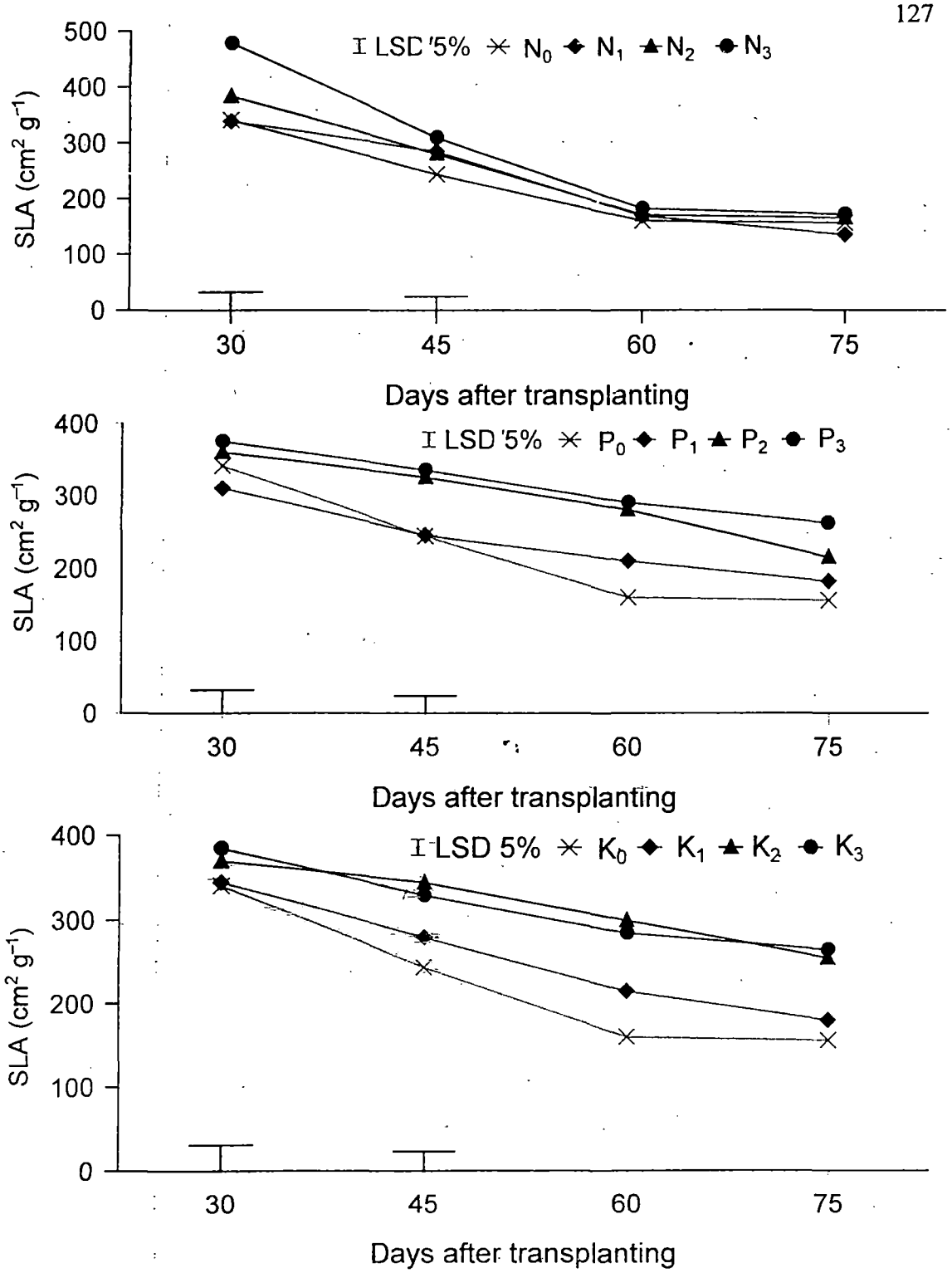


Fig: 13 Effect of different levels of N, P and K on SLA of *R. serpentina* at different stages of growth.

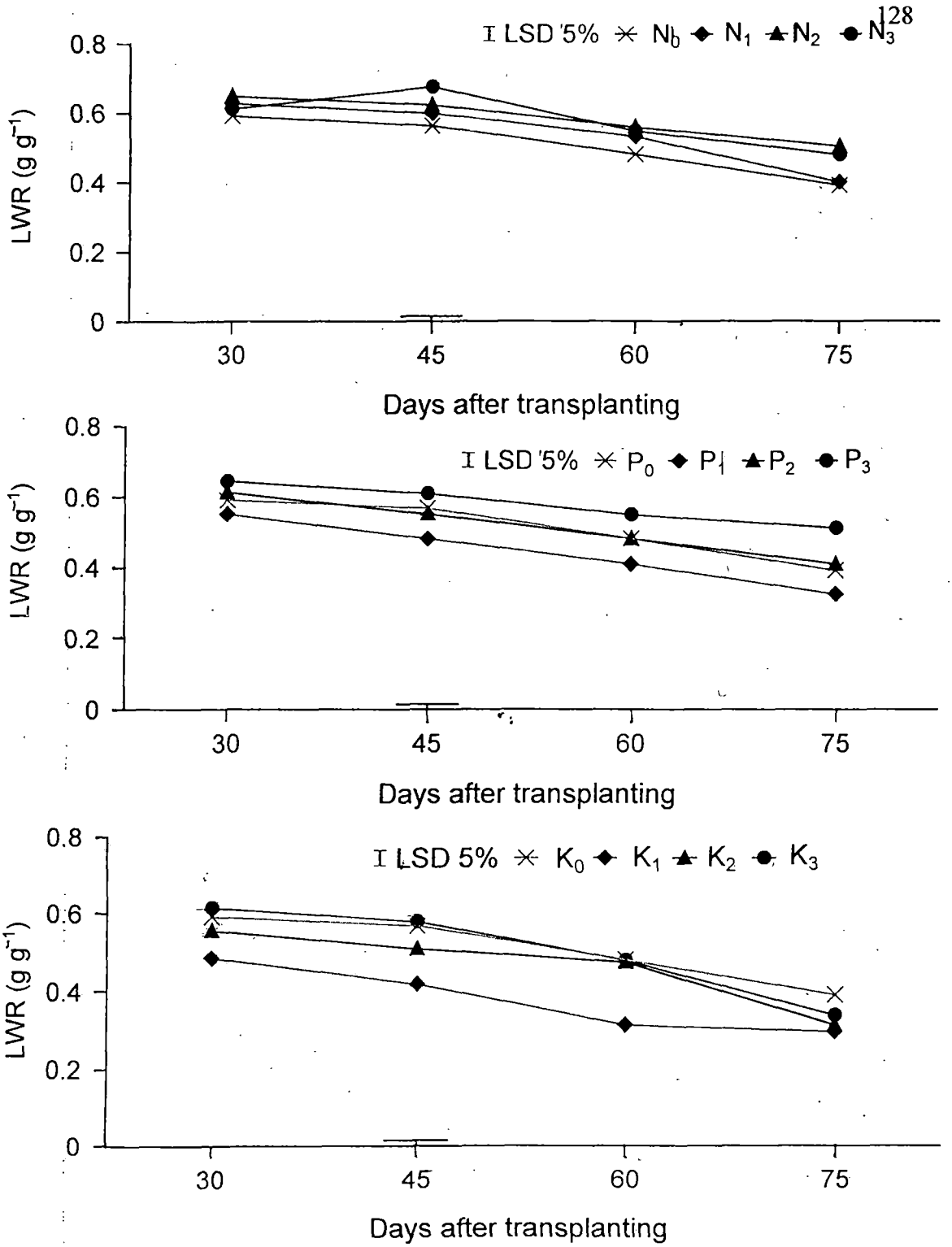


Fig: 14 Effect of different levels of N, P and K on LWR of *R. serpentina* at different stages of growth.

Chlorophyll Content

Mean values of chlorophyll a, b and total chlorophyll contents of leaf of *R.serpentina* at different levels of N, P and K are presented in Table 13. Analysis of variance indicated that the effect of N, P and K levels were significant for chlorophyll a and total chlorophyll only. Chlorophyll contents were higher in all the treatments over the control.

Compared to P and K, the different levels of N had pronounced effect on chlorophyll content. As N plays important role in chlorophyll synthesis, it is not surprising that N treated plants had higher chlorophyll content. Islam *et al.* (1988) reported that chlorophyll content in rape was increased more by N fertilizer. Paul and Sarker (1989) observed that among the NPK treatments, chlorophyll content was higher in N and NPK. Paul (1990b) also reported that rape and turnip the chlorophyll content increased with the increasing N levels.

Chlorophyll a and b are the most important pigments active in the photosynthetic process. Several workers have reported that the rate of photosynthesis in leaves is positively correlated with chlorophyll (Muramoto *et al.*, 1965; Kariya and Tsunoda, 1971). However, some workers failed to find any relationship between chlorophyll content and the rate of photosynthesis (Hesketh, 1963; Kumari and Sinha, 1972). In the present investigation, higher NAR of higher N-treated plants may be due to greater amount of chlorophyll content of those plants.

Table 13 : Effect of different levels of N, P and K fertilizers on chlorophyll content of *R. serpentina.*

Treatment	Chlorophyll a ($mg\ dm^{-2}$)	Chlorophyll b ($mg\ dm^{-2}$)	Total chlorophyll ($mg\ dm^{-2}$)
Control	2.10	0.98	3.08
N_1	2.65	1.25	3.90
N_2	3.40	1.20	4.60
N_3	3.62	1.25	4.87
Mean	2.94	1.17	4.11
Control	2.10	0.98	3.08
P_1	2.32	0.90	3.12
P_2	2.68	1.10	3.78
P_3	2.75	1.15	3.90
Mean	2.46	1.03	3.47
Control	2.10	0.98	3.08
K_1	2.08	1.00	3.08
K_2	2.78	1.15	3.93
K_3	2.80	1.08	3.88
Mean	2.44	1.05	3.49
LSD 5%	0.24	NS	0.42

Root Yield and Alkaloid Content

Although there was some increases of main root length with the increases of N and P levels, but was statistically at par with the control (Table 14). Different levels of N, P and K had significant effect on air-dried root weight (Table 14). Compared to control, P-treated plants had more root weight than N or K-treated plants. P_3 (60 kg P_2O_5/ha) had the highest root weight which was identical with N_3 (90 kg N/ha). Compared to control, N, P and K treated plants had higher total alkaloid content, but the increment was slight, statistically not significant (Table 14). No clear pattern of effect of N, P and K fertilizers was observed on reserpine content.

Dutta *et al.* (1963) reported that no significant increases in the yield of the roots of *R. serpentina* occurred as a result of fertilizer application. Sahu (1970a) noted higher dried root yield of *R. serpentina* at 45 kg N/ha. He also obtained higher root yield with P application. Saini and Mukherjee (1970) observed that in *R. serpentina*, extra application of N resulted in an increases in reproductive growth but was without any effect on root yield. A complete defloration increased root weight and also caused the plants to respond to extra application of nitrogen. The increase in root was due to a proportionate increase in stem and leaf growth caused defloration treatment. In general, an abundance of essential mineral elements, particularly N stimulated root growth but the increases in shoot growth is even more, so that the shoot to root ratio is usually higher under such conditions. In an indeterminate plant, like the one under study, more shoot growth allows for more fruit and seed production. The later not only imposes limitation on shoot growth, but also on root growth. Such a situation would not arise under complete defloration. Extra dose of fertilizer application, particularly N, would increases shoot growth unrestricted and allow for proportionate increase in root growth.

Table 14 : Root yield and alkaloid contents of *R. serpentina* as influenced by different levels of N, P and K fertilizers.

Treatment	Length of main root (cm)	Air-dried root weight/plant (g)	Total alkaloid (%)	Reserpine (%)
Control	46	37	1.32	0.24
N_1	51	44	1.57	0.28
N_2	54	48	1.60	0.34
N_3	52	56	1.82	0.32
Mean	51	46	1.58	0.30
Control	46	37	1.32	0.24
P_1	48	46	1.40	0.26
P_2	49	50	1.48	0.30
P_3	54	58	1.54	0.28
Mean	49	48	1.44	0.27
Control	46	37	1.32	0.24
K_1	42	42	1.38	0.26
K_2	45	46	1.46	0.28
K_3	48	48	1.44	0.30
Mean	45	43	1.40	0.27
LSD 5%	NS	6	NS	NS

NS = Non-significant.

Maheswari *et al.* (1988) reported that an increase in the level of N greatly increased dried root yield (maximum at 45 kg N/ha). They also reported that an increase in the level of P appreciably increased the dried root yield. The maximum

was recorded at 60 kg P/ha, beyond which it declined. Maurya *et al.* (1999) reported that 60 kg N/ha was suitable for higher root yield of *R. serpentina* in Bihar.

In the present investigation, little increase of total alkaloid content was observed with the increase of levels of N, P and K fertilizers (Table 14). The effects of fertilizers on the alkaloid biogenesis in *R. serpentina* have not been extensively studied. Nandi and Chatterjee (1975) reported that in *R. serpentina* single application of P and K could not increase the total alkaloid content as compared to untreated plants; whereas N fertilizer, both singly and in combination augmented the total alkaloid contents. They noticed maximum alkaloid synthesis in combination N:P:K and this increase in the contents of total alkaloid gained momentum with advancement of age. They also reported that alkaloid biogenesis was inversely correlated with laminar area.

Maheswari *et al.* (1988) reported that total alkaloid content in root of *R. serpentina* and its yield remained higher in 30 kg N/ha, followed by 45 kg N/ha compared with the control. But total alkaloid and their yield were higher at 60 kg P/ha. However, these differences were not statistically significant.

SUMMARY

Under plantation condition, regular supply of nutrients must be maintained for good growth and higher yields of root and alkaloid of *R. serpentina.* Effect of different combinations of NPK fertilizers on growth and yield of *R. serpentina* was studied. Five combinations of NPK fertilizers were used.

The effect of N, P and K combination on leaf area/ plant and total above ground dry weight/ plant were not pronounced at the early stages of growth, but at the

later stages both these characters showed on increasing trend with increasing N, P and K levels.

For control treatment ($N_0P_0K_0$), RGR gradually decreased with plant age, but for the remaining treatments RGR reached its peak at (45-60) days after transplanting and then declined. Similar trend was observed for NAR. Higher values of LAR was observed in the control. SLA and LWR responded to NPK in different ways at different harvests.

Simple correlation coefficients between growth attributes themselves and with root yield indicated that RGR had positive correlation with NAR at the mid-growth stage. NAR was positively correlated with LAR at the initial and the mid-growth stages. LAR had positive association with both its components, SLA and LWR. Root yield had positive correlation with both RGR and NAR.

Chlorophyll a content was higher in all the treatments over the control and gradually increased with the increase of NPK levels. There was no clear pattern of NPK fertilizers on chlorophyll b content.

Root yield and alkaloid contents were also increased by NPK fertilizers. T_4 treatment ($N_{120}P_{90}K_{60}$) produced 64.7% more root yield and 32.4% more alkaloid than that of control ($N_0P_0K_0$).

Another experiment was carried out to study the effect of different levels of N, P and K on leaf area and dry matters production, growth attributes, chlorophyll content and root and alkaloid yields of *R. serpentina.*

The treatments were 30, 60 and 90 kg N/ha, 20, 40 and 60 kg P_2O_5/ha and 15, 30 and 45 kg K_2O/ ha and control.

Both leaf area and dry weight showed an increasing trend with increasing N, P and K levels. RGR, NAR, LAR and RLGR responded much by the application of N, P and K fertilizers. The effect of different levels of N, P and K on SLA and LWR were less pronounced.

Compared to P and K, the different levels of N had pronounced effect on chlorophyll content.

Although there was some increase of main root length with the increase of N and P levels, but was statistically at par with the control. Compared to control, P-treated plants had more root weight than the N or K treated plants. P_3 (60 kg P_2O_5/ ha) had the highest root weight, which was identical with N_3 (90 kg N/ ha). Compared to control, N, P and K-treated plants had higher total alkaloid content, but the increment was slight. No clear pattern of effect of N, P and K fertilizers was observed on reserpine content.

CHAPTER 5

IRRIGATIONAL EFFECT ON SOME PHYSIOLOGICAL CHARACTERS AND ROOT AND ALKALOID YIELDS OF *R. serpentina.*

INTRODUCTION

R. serpentina grows wild in forests under high rainfall conditions of about 1,000 mm to 1500 mm per annum. The natural habitat of the plant suggests that it is grown in partial shaded areas as an undergrowth. As a result, plant water deficit usually increases less quickly and photosynthesis proceeds for longer period in shelter, so allowing better dry matter production and increased efficiency in water use. A prerequisite for the field cultivation of *R. serpentina* is to create forest microclimate that prevails at the habitat of the plant. The moist humid micro-climate favourable and necessary for this undergrowth in forest can be provided in the open field by means of adequate irrigation.

Rainfall is the major component of water resources in India and provide a major share of the crop-water requirements during the wet season and partially during the dry season through storage and soil layers. During the winter season rainfall occurs very rarely. Therefore, if *R. serpentina* field is not irrigated, growth is restricted due to water stress. Information is needed regarding the physiological and morphological responses of *R. serpentina.* Little information is available on the irrigation and water requirement of *R. serpentina* as a plantation crop. Increasing

existing knowledge of the nature of the response to drought and / or irrigation will promote higher production potential and more efficient water use in *R. serpentina*.

Developmental factors influencing the accumulation of dry matter and subsequent partitioning of assimilates are of great importance in determining the final yield of crop plants (Watson, 1971; Wareing and Patric, 1975). Water is one of the most essential factors influencing plant growth and development. A number of workers reported significant increases of dry matter by water application in a number of crop plants [Krogman and Hobbs (1975) and Kundu and Paul (1995-96) in rape, D'Souza and Coulson (1988) in *Phaseolus vulgaris*, Mondal and Paul (1992) and Begum and Paul (1993) in mustard and Sarker and Paul (1998), Nahar and Paul (1988) in wheat]. Several workers have reported that irrigation had positive influence on growth parameters such as RGR, NAR, LAR, RLGR, SLA and LWR in a number of crop plants (Sarker and Paul, 1998; Nahar and Paul, 1998; Sarker *et al.*, 1996; Nerkar *et al.*, 1981; Mondal and Paul, 1995; Saha and Paul, 1995; Mondal and Paul, 1994).

Fischer (1973) found that relative water content was directly related to soil water content and suggested that relative water content might also be used to indicate soil water content. Free proline accumulation occurs in the leaves of crop plants when exposed to moderate to severe water stress (Palfi *et al.*, 1973). Free sugars are also accumulated in the stressed plants (Stewart, 1971; Narashima Rao and Shiv Raj, 1985). It has been suggested that proline and sugar accumulating potentials could serve as indices of drought resistance. However, the physiological significance of metabolic response to water stress is contentious.

Since scientific data on this aspect of drought resistance are meagre, an attempt was made to study the effect of soil moisture on relative leaf water content

(RLWC) and some biochemical parameters, such as chlorophyll, proline and sugar contents of *R. serpentina*. Soil moisture effect on root yield and alkaloid content was also studied.

MATERIALS AND METHODS

The experiment was carried out in the experimental field of North Bengal University. The field was prepared after repeated ploughing. A basal dose of urea (80 kg/ ha), TSP (50 kg/ ha) and MP (40 kg/ ha) were added to the field. Uniform and healthy seedlings of *R. serpentina* of 30–35 days old were transplanted in the field by the end of September, 1998. A spacing of 30 cm x 30 cm was followed and three were 3 replications.

Three levels of irrigation treatment were adopted, viz., (i) rainfed (I_0 = no irrigation), (ii) irrigation once in every month (I_1) and (iii) irrigation twice in every month (I_2). On each occasion, 20 mm of irrigation water were uniformly added over the irrigated plots with sprinklers. Irrigation started from November, 1998 and continued upto May, 1999.

Growth Attributes

For growth analysis, four harvests were taken at equal interval of 15 days. Three plants/ treatment/ replication were taken on each occasion. The first harvest was taken at 60 days after transplanting (DAT). At each harvest, plants were cut at the ground level and the tops were separated into leaves, petioles and stems. The dry weights of different plant parts were recorded after oven-drying at about 85°C for 24

h till they reached constant weight. For leaf area measurement, disc method was followed. From leaf area and dry weight data, different growth attributes were calculated as in Chapter 4.

Relative Leaf Water Content (RLWC)

Relative leaf water content was determined from the fully matured leaves. The leaves were collected at 8 a.m., 12 noon and 4 p.m. Three leaves were taken from each replication of each treatment. Their fresh weights were taken immediately and were sunk into water kept in beaker for 4 hours. After 4 hours, when the cells of the leaves became fully turgid, they were taken out from water and after drying with blotting paper their turgid weights were determined. Then the leaves were dried in an oven and weighed. The RLWC was calculated from the following formula (Barrs and Weatherley, 1962) :

$$RLWC = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

Chlorophyll Content

Chlorophyll content of the matured leaf was determined as in Chapter 4.

Estimation of Proline

Proline estimation of the matured leaves was done according to Bates *et al.* (1973). For estimation of proline, the reagents required were (1) 3% aqueous sulphosalicylic acid, (2) Glacial acetic acid, (3) Tolune, (4) Proline (AR) and (5) Acid ninhydrin reagent [6M orthophosphoric acid (20 ml) + glacial acetic acid (30 ml) + ninhydrin (1.25 g)].

At first 0.5 g fresh leaf was homogenized in 10 ml of 3% aqueous sulphosalicylic acid and centrifuged it for 6 minutes. The clear solution was separated and 2 ml of it was reacted with 2 ml of acid nynhydrin and 2 ml glacial acetic acid in a test tube. Then it was boiled in a boiling water bath for 1 hour and the reaction was terminated in an ice bath. The reaction mixture was then extracted with 4 ml of toluene mixed vigorously with stirring for 15 – 20 seconds. The chromophore containing proline-toluene was separated with a separating funnel and warmed to room temperature. The optical density ($O.D._{.}$) at 520 nm was read using toluene as blank. Proline content was determined from the standard curve.

Stock solution of 1 millimole proline was prepared by dissolving 0.1151 g of proline (AR) in distilled water and made to 1000 ml. By successive dilution 2 ml of solution containing 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 micromole concentrations were prepared and reacted with acid ninhydrin and glacial acetic acid as described earlier. The colour was read at 520 nm and the O.D. was plotted against concentration (Figure 15).

Determination of Sugar Content

Extraction of sugar from matured leaves was done following the method described by Jayaraman (1975).

One gram of leaf was thoroughly crushed in a mortar with a pestle and 10 ml of ethyl alcohol were used for leaf crushing. The extract was filtered through two layers of muslin cloth. The volume of the extract was evaporated to about one-fourth of the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100 ml volumetric flask and made up to the mark with distilled water. One ml of the diluted solution was taken into another 100 ml volumetric flask and made up to the mark with distilled water.

Aliquot of 1 ml of the extract was pipetted into test tubes and 4 ml of anthrone reagent was added to each of these solutions and mixed well. Glass marbles were placed on top of each tube and the test tubes were heated for 10 minutes in boiling water bath and then cooled. A reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured at 680 nm in a spectrophotometer.

A standard curve of glucose was prepared by taking 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard glucose solution in different test tubes containing 0.0, 10, 20, 40, 60, 80 and 100 µg of glucose, respectively and made the volume up to 1.0 ml with distilled water. Four ml of anthrone reagent were added to each tube and mixed well. All these solutions were treated similarly as described above. The absorbance was measured at 640 nm using the blank containing 1 ml of water and 4 ml of anthrone.

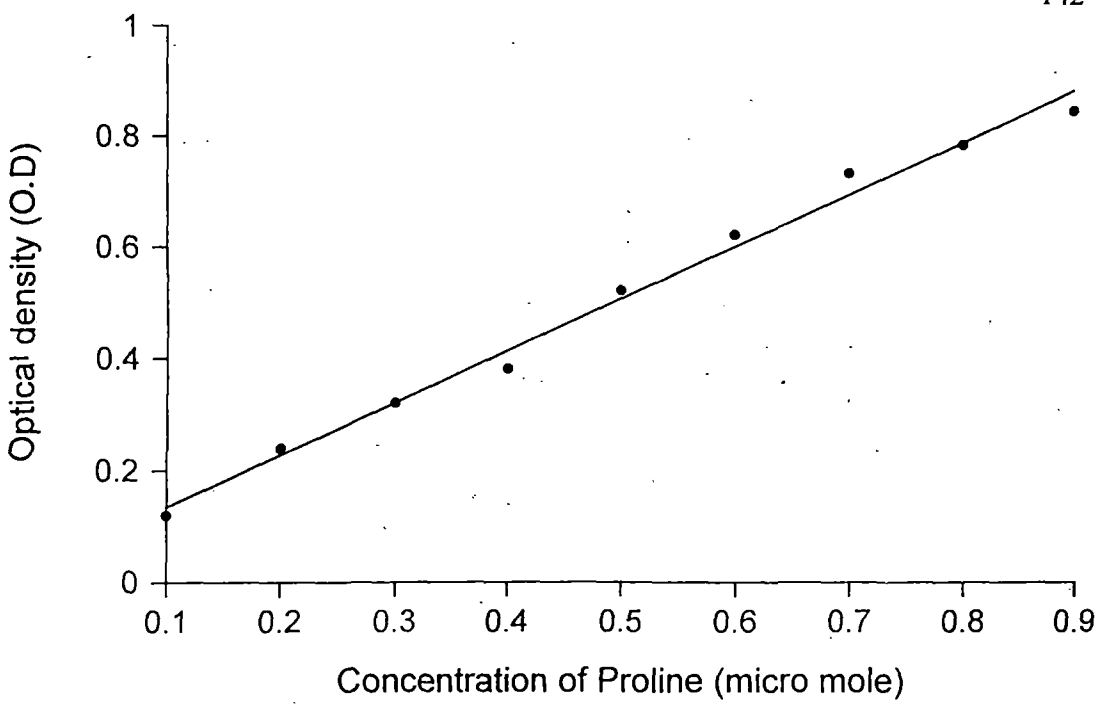


Fig. 15 Standard curve for proline

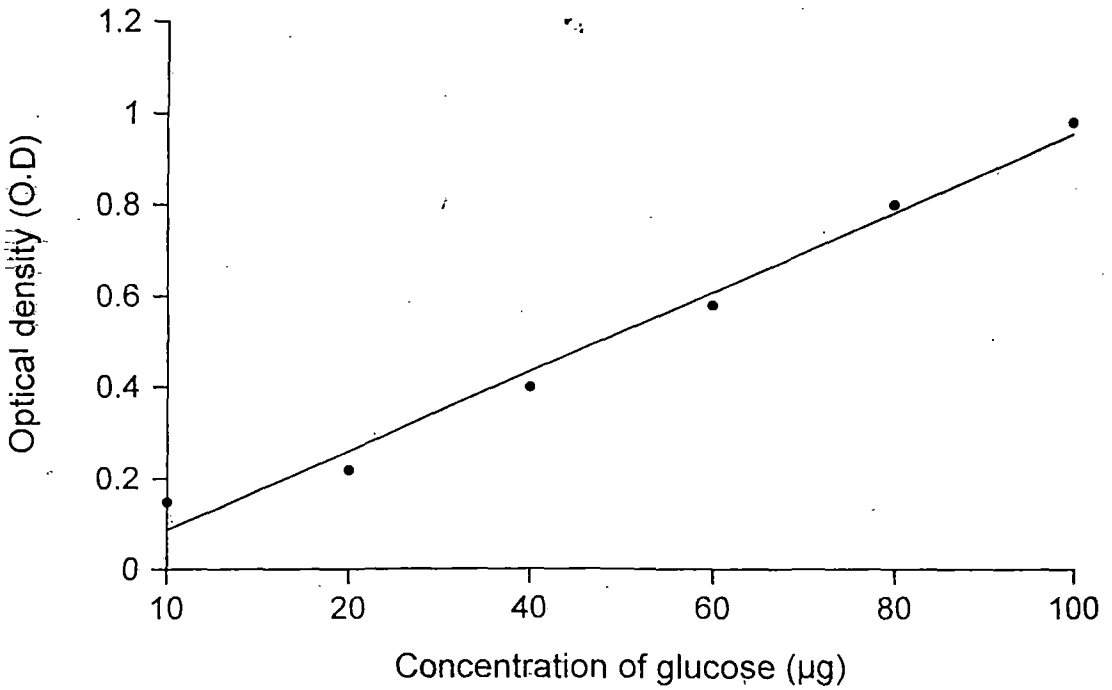


Fig. 16 Standard curve for glucose

The amounts of free sugar were calculated from the standard curve of glucose (Figure 16). Finally the percentage of free sugar present in the leaves was determined using the formula given below:

$$\text{Percentage of free sugar} = \frac{\text{Weight of sugar obtained}}{\text{Weight of leaf dust}} \times 100$$

Sugar content was determined at 3 times i.e. at 120, 180 and 240 days after transplanting.

Root Yield and Alkaloid Content

After 18 months of planing, plants were dug out in March, 2000 and the roots were separated and washed in the running tap water. The roots were air-dried to a constant weight. Six randomly selected plants from each treatment and replication were used for this purpose. Composite root samples were analysed for total alkaloid and reserpine contents as in Chapter 2 of this study. Data were analysed statistically.

RESULTS AND DISCUSSION

Leaf Area and Dry Matter

Effect of soil moisture on leaf area and total dry matter of *R. serpentina* is shown in Figures 17 and 18, respectively. Both leaf area and dry matter were significantly affected by soil moisture. Compared to control, I_1 and I_2 treatments had greater values at all the stages of growth. Similar result was reported in several plants

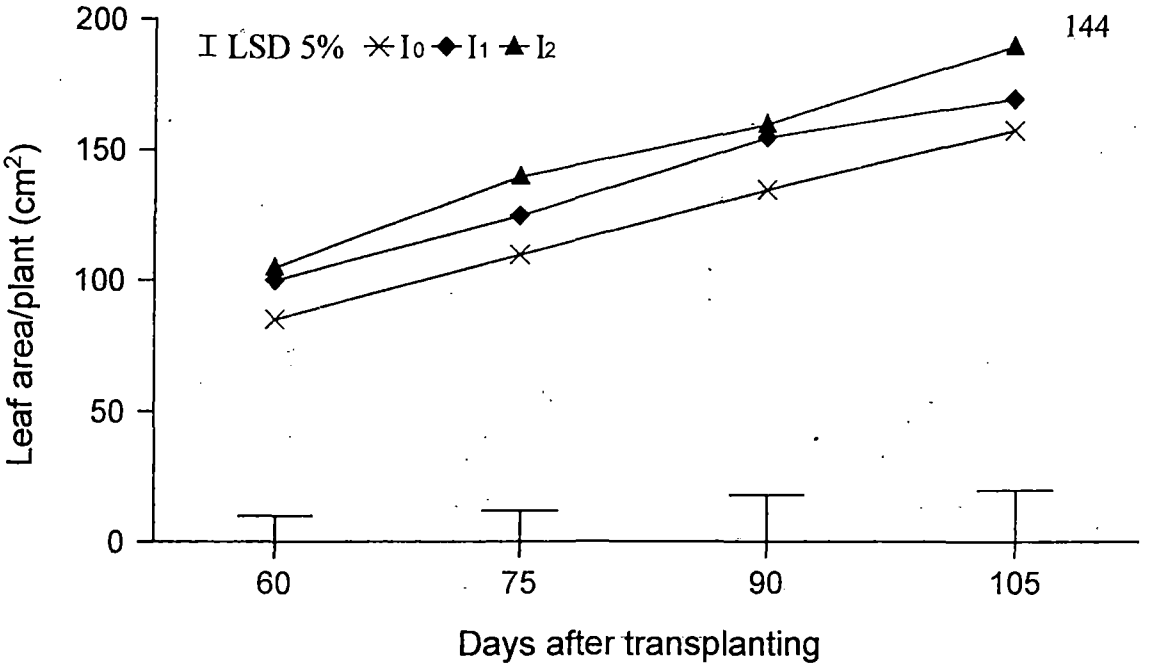


Fig. 17 Influence of soil moisture on leaf area of *R. serpentina*

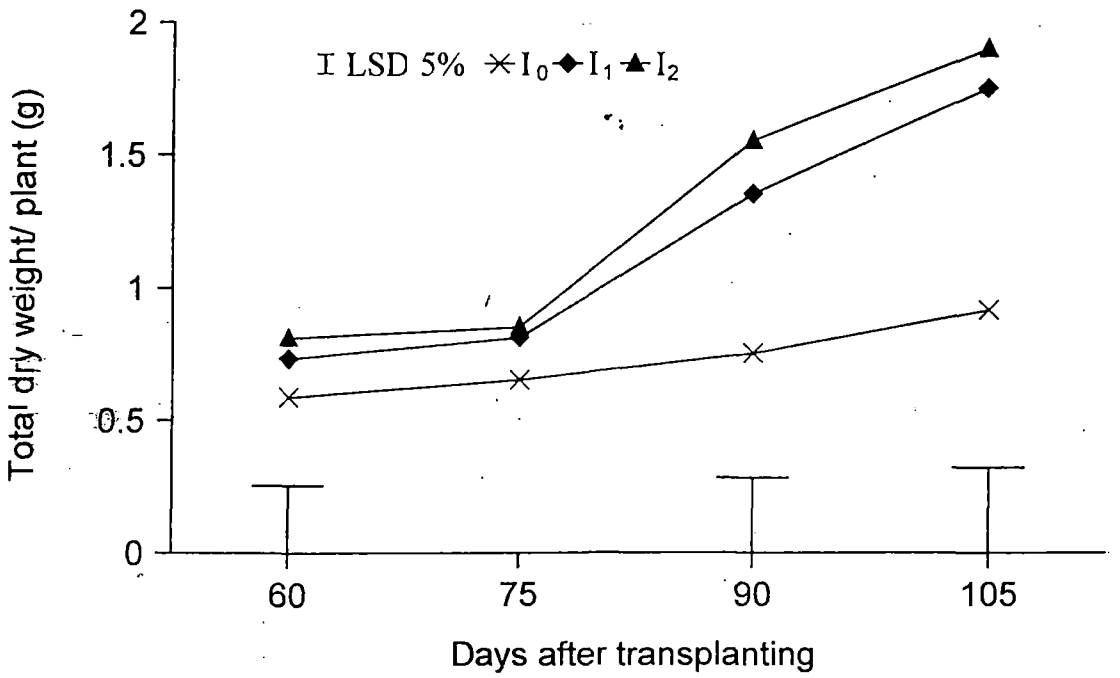


Fig. 18 Influence of soil moisture on total dry weight of *R. serpentina*

like barley (Kirby, 1969), sorghum (Constable and Hearn, 1978; Sivkumar *et al.*, 1979 and Rabindranath and Shiv Raj, 1983), mustard (Mondal and Paul, 1992, 1994, 1995; Begum and Paul, 1993), in rape (Clark and Simpson, 1978; Paul and Kundu, 1991) and in wheat (Rahman and Paul, 1998; Nahar and Paul, 1998; Sarker and Paul, 1998).

Leaf area of plant may be varied due to change either in leaf number or leaf size (Arnon, 1975). The increase of leaf area occurred due to increase of the leaf expansion in the irrigated plants. Soil moisture increased turgor pressure in the cells and turgor forces played a part in the process of leaf expansion (Hsiao and Acevedo, 1974). With the increase in the frequency of irrigation, uptake of nutrients was more, hence more expansion of leaf took place (Mandal *et al.*, 1986).

Soil moisture increased the relative leaf was content, which increased cell expansion and ultimately leaf area increased. Mare and Palmer (1976) found that the total number of leaves produced by the primary stem of sunflower was reduced due to water stress. This may be due to the inhibition of the initiation and differentiation of leaf primordia due to water stress.

Growth Attributes

Compared to control, RGR of the irrigated plants was higher at the initial and the final harvest intervals, but lower at the middle harvest interval (Figure 19). Saha and Paul (1995) studied the effect of soil moisture on growth attributes of wheat and reported that RGR was unaffected by soil moisture and it decreased with plant age. Richards (1978) found that RGR was reduced in more severe drought treatment in rape.

RGR declined with increasing age and plant dry weight. Similar results were reported for RGR in barley (Thorne, 1960) and in wheat (Sarker and Paul, 1998; Nahar and Paul, 1998; Sarker *et. al.*,.1996). It had been suggested that the decrease in RGR could be attributed to shading of lower leaves by upper leaves (Thorne, 1961). The decreasing trend in RGR with age was mainly due to decline of LAR (Chanda *et al.*, 1987).

Irrigation increased NAR (Figure 20). Higher NAR due to higher soil moisture was found by El Nadi (1969) and Nerkar *et al.* (1981) in beans and Rabindranath and Shiv Raj (1983) in sorghum. Unlike RGR, NAR in all the treatments increased with plant age and weight. As *R. serpentina* is a long duration crop, NAR did not decrease up to 105 DAT. But LAR decline steadily with increasing plant age (Figure 21). Similar results were reported in dry beans by Wallace and Munger (1965), in wheat by Sarker and Paul (1998) and Nahar and Paul (1998), in mustard by Begum and Paul (1993) and Mondal and Paul (1994). In the present investigation, irrigated plants had lower LAR than the rainfed control. But Sarker and Paul (1998) observed slightly higher LAR in the irrigated treatments. Kirby (1969) also reported that LAR increased with increasing level of irrigation in barley. Similar result was reported in bean by Nerkar *et al.* (1981). However, no significant effect of irrigation on LAR was found by Mondal and Paul (1992), but Paul and Kundu (1991) and Mondal and Paul (1995) reported decreased LAR in rape and mustard, respectively.

The plants under irrigated condition had higher RLGR than the rainfed ones (Figure 22). Similar result was reported in wheat (Saha and Paul, 1995; Sarker and Paul, 1998). But Mondal and Paul (1995) did not find any significant effect of soil moisture on RLGR of mustard. RLGR in all the treatments declined with increasing

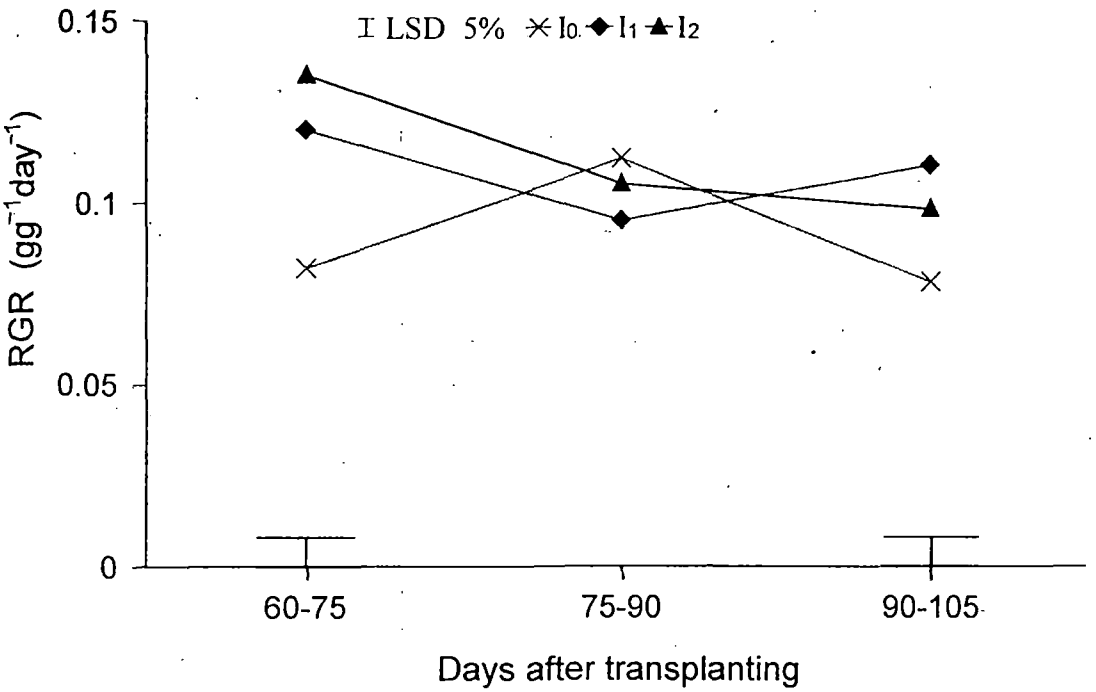


Fig. 19 Influence of soil moisture on RGR of *R. serpentina*

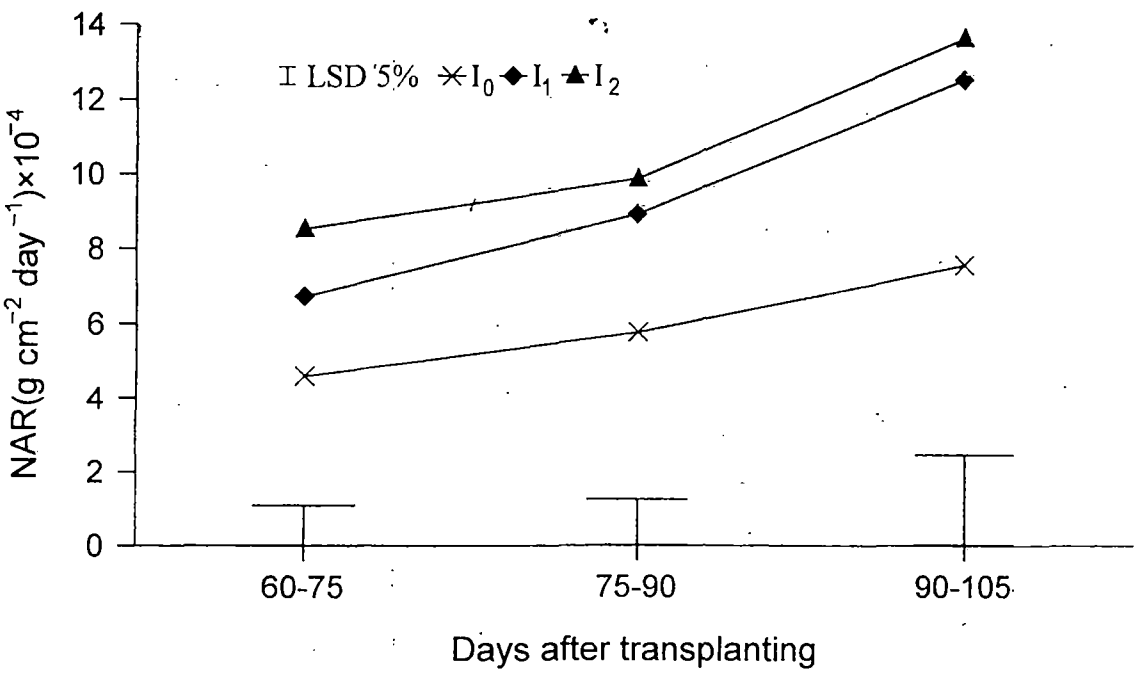


Fig. 20 Influence of soil moisture on NAR of *R. serpentina*

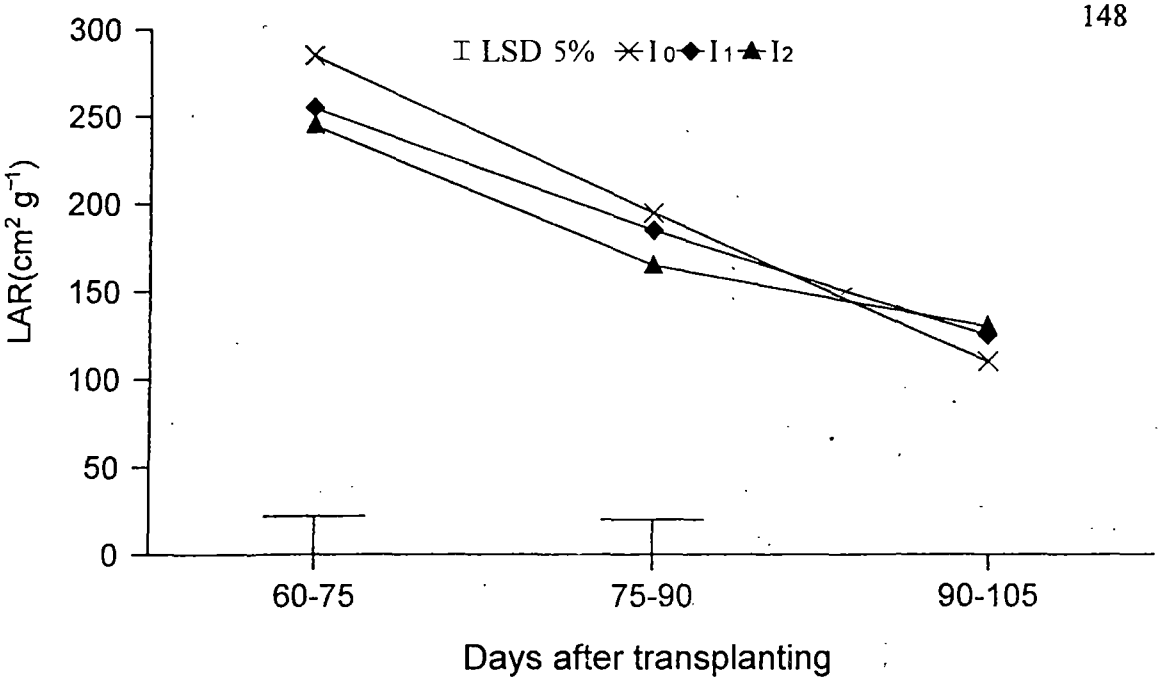


Fig. 21 Influence of soil moisture on LAR of *R. serpentina*

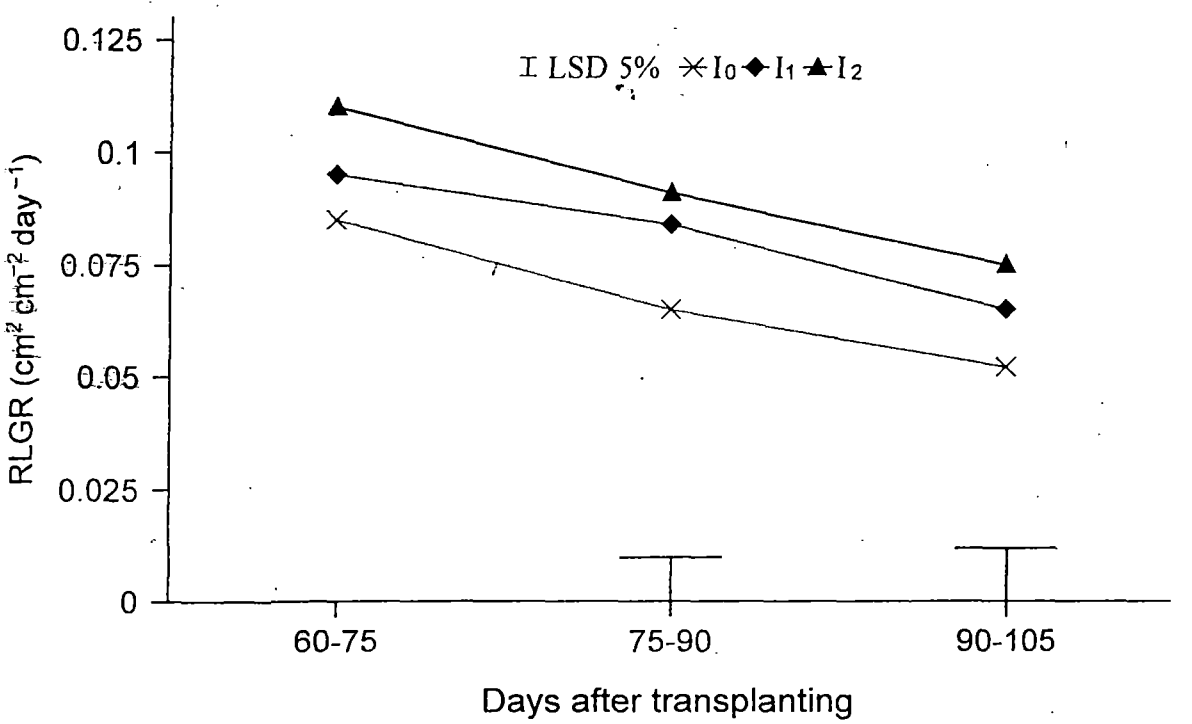


Fig. 22 Influence of soil moisture on RLGR of *R. serpentina*

plant age. Similar decline in RLGR with plant age was reported by Pandey *et al.* (1978) in blackgram and Chanda *et al.* (1987) in pearl millet.

There were no clear patterns of soil moisture effect on SLA (Figure 23), although there was some tendency of increase of SLA with increasing soil moisture. Saha and Paul (1995) and Nahar and Paul (1998) indicated that SLA was decreased by irrigation. Paul and Kundu (1991) found that SLA decreased by irrigation in rape. But Mondal and Paul (1995) did not find significant effect of irrigation on SLA. In the present investigation, SLA gradually decreased with plant age. Decrease of SLA with plant age was reported in wheat (Saha and Paul, 1995; Sarker and Paul, 1998 and Nahar and Paul, 1998), in mustard (Islam and Paul, 1986), in jute (Hussain and Paul, 1984) and in pearl millet (Chanda *et al.*, 1987).

Like SLA, there was no clear pattern of soil moisture effect on LWR (Figure 24). But Sarker and Paul (1998) in wheat and Mondal and Paul (1995) in mustaerd reported that LWR was higher in the well-watered plants. The values of LWR showed downward drifts with age (Figure 24). Similar result was reported by Mondal and Paul (1995) in mustard, Sarker and Paul (1998) in wheat and Chanda *et al.* (1987) in pearl millet. Similar results were also reported in sweet potato (Shamsuddin and Paul, 1988) and in jute (Hussain and Paul, 1984). Kundu (1992) stated that the sharp decrease of LWR at the later stages might be due to sharp increase of total dry matter towards the later stages.

Relative Leaf Water Content

Relative leaf water content (RLWC) of the irrigated plants (I_1 and I_2) was significantly higher than that of the rainfed plants (I_0) (Table 15). Similar result was

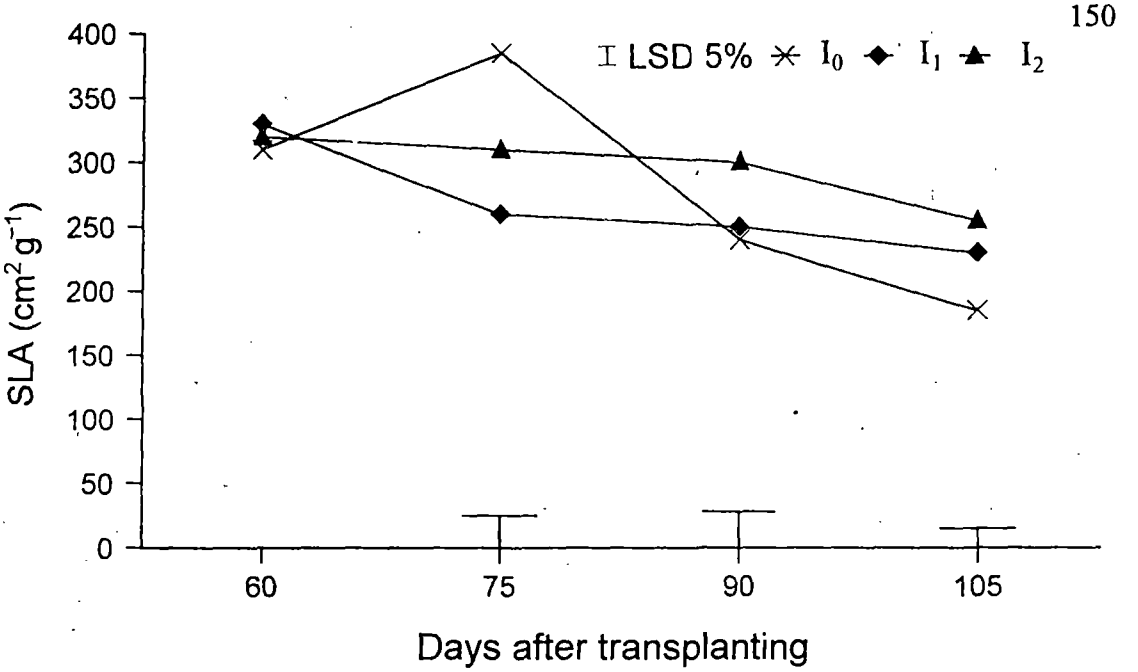


Fig. 23 Influence of soil moisture on SLA of *R. serpentina*

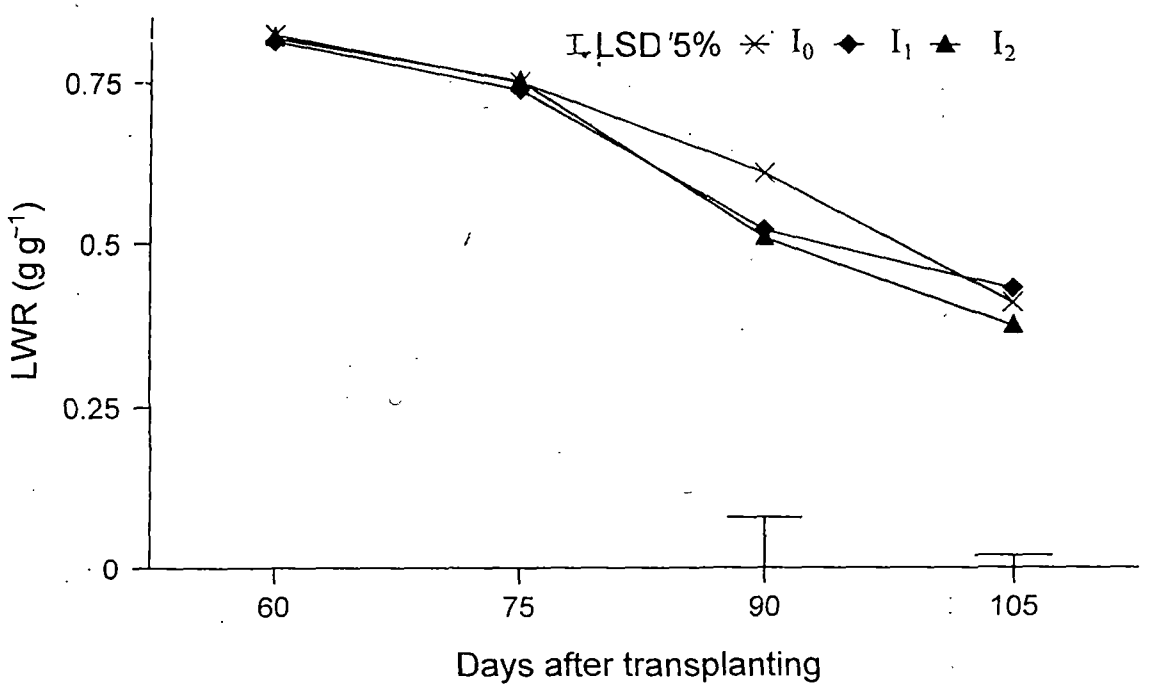


Fig. 24 Influence of soil moisture on LWR of *R. serpentina*

reported in wheat by Rajagopal et al., (1977), Nayak *et al.* (1983), Sarker *et al.* (1999), Schonfeld *et al.* (1988), Rahman and Paul (1998), in rape by Raja and Bishnoi (1990) and Kundu and Paul (1996) and in mustard by Sharma and Kumar (1989) and Begum and Paul (1993).

Table 15 : Relative leaf water content(%)of *R. serpentina* at different times of the day as influenced by soil moisture.

Treatment	Time		
	8 a.m.	12 noon	4 p.m.
I_0	88.0	75.0	75.8
I_1	93.2	79.3	82.0
I_2	95.7	80.0	84.7
LSD 5%	3.2	2.7	4.0

In all the three treatments, RLWC was higher towards the morning, got reduced at noon and again showed some recovery towards the afternoon (Table 15). This behaviour was related to the environmental conditions and is in agreement with earlier reports (Begum and Paul, 1993; Mondal and Paul, 1996; Sarker and *et al.*, 1999). At midday, RWLC of the rainfed plants (I_0) decreased more than that of the irrigated plants (I_1 and I_2). Cortes and Sinclair (1986) also found more negative leaf water potential of stressed soybean plants than that of the irrigated ones at midday. Higher RWLC is associated with higher dry matter production rates of the irrigated plants because cell turgidity is important in relation to the opening and closing of stomata, expansion of leaves and movement of nutrients of various parts of the plant (Kramer, 1969).

Chlorophyll Content

Chlorophyll a and b are the most important pigments active in the photosynthetic process. Increased chlorophyll contents absorb higher quantity of light and hence increased photosynthesis. Several workers have reported that the rate of photosynthesis in leaves is positively associated with chlorophyll (Muramoto *et al.*, 1965; Kariya and Tsunoda, 1971). Hesketh (1963) showed that species could vary greatly in rate of photosynthesis and this variation was not related to chlorophyll content. Buttery and Buzzell (1972) reported that many crop plants were never light saturated even in full sunlight and that a higher level of chlorophyll could further increase light absorption and photosynthetic rate only if sufficient light was provided.

In the present investigation, soil moisture effect was found to be significant for chlorophyll a, b, and total chlorophyll (Table 16). A reduction of chlorophyll formation due to water stress was also reported by Mondal and Paul (1992), Begum and Paul (1993), Kundu and Paul (1997) and Sarker *et al.* (1999). However, Saha and Paul (1997) did not find any significant effect of soil moisture on chlorophyll content of wheat.

Table 16 : Mean values of chlorophyll content (mg dm^{-2}) of *R. serpentina* leaf as influenced by soil moisture.

Treatment	Chlorophyll a	Chlorophyll b	Total chlorophyll
I_0	3.07	0.72	3.79
I_1	3.62	0.87	4.48
I_2	3.71	0.90	4.61
LSD 5%	0.13	0.09	0.14

Proline Content

Proline works as a source of energy, carbon and nitrogen and also protects several enzymes against the inactivating effects of heat during water stress (Paleg *et al.*, 1981). Hence, possibly proline accumulation under water stress helps the plant to resist the drought. In this study, the rainfed plants (I_0) had significantly higher proline content than the irrigated plants (I_1 and I_2) (Table 17). Similar result was also reported in wheat (Saha and Paul, 1997; Rahman and Paul, 1998; Sarker *et al.*, 1999), in rape (Paul and Kundu, 1991; Kundu and Paul, 1997), in mustard (Begum and Paul, 1993), in sorghum (Blum and Ebercon, 1976) and in foxtail millet (Narashima Rao and Shiv Raj, 1985). Total free amino acids are increased in water stressed leaves and proline being the most pronounced (Hsiao, 1973).

Table 17 : Proline content (μg/g fresh weight) of *R. serpentina* leaf as influenced by soil moisture at different stages of growth.

Treatment	Days after transplanting		
	120	180	240
I_0	249	265	272
I_1	166	170	180
I_2	148	174	191
LSD 5%	24	32	28

Blum and Ebercon (1976) suggested that the accumulated proline could also serve as a readily available energy and nitrogen source for use upon the relief of stress. Accumulated proline may be oxidized and serve as a source of energy, espaecially when carbohydrate content is low (Wang, 1968; Oks *et al.*, 1970; Stewart, 1971). There is also evidence that proline may confer protection on some mitochondrial and solubilized enzymes against heat instability (Nash *et al.*, 1980).

In the present investigation, accumulation of proline in all the treatments increased with the advancement of plant age (Table 17). Similar result was reported by Kundu and Paul (1997) in rape. This increase might be due to increased atmospheric and soil drought at the later stages of growth.

Total Sugar Content

Total free sugar content was significantly (except at 240 DAT) higher in the rainfed plants at all the three stages of growth (Table 18). Turner *et al.* (1985), Drossopoulos *et al.* (1987), Kundu and Paul (1997) and Sarker *et al.* (1999) obtained similar results. As the stress increased at the later stage, amount of sugar also increased in all the three treatments (I_0, I_1 and I_2), but the amount was much greater in the rainfed plants.

Plants accumulated more sugar during stress which can be utilized when stress is released (Stewart, 1971). Easten and Ergle (1948) suggested that starch would be converted into sugar under stress. Sugar is also known to stabilize protoplasmic membranes (Larson, 1975).

Table 18 : Total sugar content (mg/ g dry weight) of leaf of *R. serpentina* at three growth stages as influenced by soil moisture.

Treatment	Days after transplanting		
	120	**180**	**240**
I_0	12.6	13.5	14.8
I_1	11.7	11.5	12.4
I_2	9.5	9.8	11.5
LSD 5%	1.7	2.5	NS

The present study suggests that moisture stress had an inhibitory effect on RLWC and chlorophyll content, but proline and sugar contents of leaves increased markedly as a result of soil dryness. High proline and sugar accumulation of *R. serpentina* in drought conditions might be an adaptation for tolerance of drought which helps the crop to survive and continue production during the dry season. The above parameters may also be used to assess crop water status.

Root Yield and Alkaloid Content

Effects of soil moisture on root yield and alkaloid contents of *R. serpentina* are shown in Table 19. Results indicated that main root length, total alkaloid and reserpine contents were unaffected by soil moisture, but air-dried root yield gradually increased with the increase of irrigation frequency.

Table 19 : Effect of soil moisture on root yield and alkaloid contents of *R. serpentina*

Treatment	Main root length (cm)	Air-dried root weight/ plant (g)	Total alkaloid content (%)	Reserpine content (%)
I_0	50	32	1.62	0.38
I_1	52	37	1.83	0.42
I_2	47	44	1.74	0.40
LSD 5%	NS	3	NS	NS

Little information is available on the irrigation and water requirement of of *R. serpentina* as a plantation crop. Sahu (1972) reported a field experiment at the State Research Station, Bhubaneswar, Orissa. Irrigation started from January when the plantion was about four and a half month old. The results revealed that the longest root of 55 cm length was noticed in plants receiving no irrigation and the shortest root of 45 cm length was in plants receiving irrigation every month. Plants received irrigation in alternate month showed intermediate growth of 50 cm long. He further reported that overall growth of *R. serpentina* plantation diminished with increasing water stress but root growth was less influenced than was shoot growth. Root yield of the crop grown without irrigation was less than the irrigated ones. Irrigating the plantation once in every month from January to May raised the root yield by about 54.3%. Irrigation in alternate months also increased root yield by about 22.6%. Withholding irrigation in February, the yield of roots was decreased by 8.36% in comparison with the yield of the crop receiving irrigation in that month. He further reported that for a 18 months old *Rauvolfia* plantation. 139 mm water provided by 5 irrigations is needed to get high root yield.

The results of the present investigation indicated that better growth of *R. serpentina* is possible in well watered condition which ultimately considered as the basic need for satisfactory root yield. The results also suggest that during the dry season, irrigation of *R. serpentina* once in a month will assure high root yield.

The overall results of the present study suggested that leaf area and dry matter production were significantly increased by soil moisture. Higher dry matter and root yield of the irrigated crop were due to the increase of net assimilation rate. Increased chlorophyll content also contributed to higher NAR of the irrigated crop. Relative leaf water content (RLWC) of the irrigated crop was higher than the rainfed crop. The RLWC was associated with higher dry matter production rates because cell turgidity is important in relation to the opening and closing stomata, expansion of leaves and movement of water and nutrients to various parts of the plant. Physiological criteria like NAR can be taken as a criterion for root yield improvement of *R. serpentina* under irrigated condition.

SUMMARY

Information is needed regarding the morphological and physiological responses of *R. serpentina* to water stress for cultivation as plantation crop. The effect of soil moisture on growth parameters, relative leaf water content (RLWC), chlorophyll, proline and sugar contents and root and alkaloid yields was studied. Three levels of irrigation treatment were adopted: rainfed, irrigation once in every month and irrigation twice in every month.

Both leaf area and dry matter/ plant were significantly increased with increase of soil moisture. Compared to the control, RGR of the irrigated plants was higher at the initial and the final harvest intervals, but lower at the middle harvest interval.

Irrigation increased NAR. The plant under irrigated condition had higher RLGR than the rainfed ones. There were no clear patterns of soil moisture effect on SLA and LWR.

Relative leaf water content of the irrigated plants was significantly higher than that of the rainfed plants. Chlorophyll content was higher in the irrigated plants but proline content was higher in the rainfed control. Total free sugar content was also significantly higher in the rainfed plants at all the three stages of growth.

Effect of irrigation on main root length, total alkaloid and reserpine contents were unaffected by soil moisture, but air-dried root yield increased with the increase of irrigation frequency.

CHAPTER 6

TISSUE CULTURE IN *R. serpentina*

INTRODUCTION

Plant tissue culture is the technique of culturing isolated cells, protoplast or organs viz., leaf, root, anther, ovule, embryo, meristem, shoot tip etc. under aseptic condition in an appropriate medium. During the last few decades the tecniques of plant cell and tissue culture have opened up several new possibilities and developed as a powerful tool for crop and ornamental plant improvement and received wide attention of modern scientists (Carlson, 1975; Razdan and Cocking, 1981; Vasil and Vasil, 1980). Tissue culture techniques are becoming increasingly popular as an alternative means of plant vegetative propagation. The most popular application of plant tissue culture is micropropagation.It represents the optimum efficiency in terms of vegetative plant propagation and allows large number of propagules to be produced in a relatively short period of time under controlled conditions throughout the year in a relatively small space.

The technology owes its origin to the ideas of the German plant physiologist Haberlandt (1902), who in his famous address to the German Academy suggested that it should be possile to cultivate artificial enbryos from vegetative cells. Although he was not successful in his attempts,he introduced the concept that all living cells containing normal complement of chromosomes should be capable of regenerating the entire plant that we recognize today as totipotency. The indepencent and pioneering studies of Gautheret (1939), Nobe Court (1939) and White (1939)

demonstrated the proliferative capacities of cells in culture. These findings set the stage for the large increase in research undertaken during the 1940s, 1950s and 1960s.

In vitro micropropagation has a number of advantages over the sexual one in a large-scale propagation programme (Abbott, 1978; Vasil and Vasil, 1980; Bonga, 1982). These advantages are: (1) superior gene combinations are propagated unaltered by cloning superior plants, which are lost through gene recombination in sexual method; (2) improvement of gene pool of planting stock in sexual means is slow because of long breeding cycle, whereas with cloning it is easy and much faster to get a true – to – mother type of a desirable clone; (3) genetic uniformity of a clone is maintained; (4) in some species, clonal propagules initially grow much faster than seedlings; (5) often the juvenile phase of development can be bypassed if desired and some valuable hybrid and polyploid plants are infertile in general, but can be propagated by cloning through tissue culture; (6) in relatively short time and space a large number of plants can be produced starting from a single individual and can be continued all the year round.

In the recent trends of plant research rapid multiplication has gained considerable importance as a promising tool for propagation of medicinal plants and it has already been possible to increase certain types of alkaloids from *in vitro* grown medicinal plants (Butcher *et al.*, 1971; Kamada *et al.*, 1971; Harkes *et al.*, 1985; Verporte *et al.*, 1985; Heizden *et al.*, 1986; Elert *et al.*, 1986; Payne *et al.*, 1987; Walton *et al.*, 1988; Mathur *et al.*, 1990)

There were previous attempts for the propagation of *R. serpentina* through tissue culture. Mitra *et al.* (1985) obtained the leaf callus cultures of *R. serpentina* by activating the phloem and parenchyma around the vascular region of the leaf lamina. Callus cultures from leaf of *R. serpentina* were also reported by Mitra and

Chutturvedi (1970) and the studies were confined to histogenesis and regeneration. Ilahi and Akram (1987) studied the various media and growth hormones with two types of leaf explants of *R. serpentina* for callus initiation, proliferation of leaf callus and their chemical analysis. Techniques for vegetative propagation of *R. serpentina* through bud differentiation in stem callus had been developed (Akram and Ilahi, 1985) and root callus of *R. serpentina* was induced to differentiate soil transferable plantlets.

Callus tissue of *R. serpentina* from leaf, stem and root were subjected to screening for 5 important alkaloids (Ilahi *et al.*, 1988). The major alkaloid found was ajmaline; serpentine and raubasine were ascertained quantitatively and reserpine and raupine were found in traces only. The percentage of ajmaline for stem callus was more than that found in stems of cultivated plants, whereas in leaves of cultivated plants ajmaline was found in traces. For root callus the quantity of ajmaline observed in cultures was a bit lower than in cultivated plants.

In tissue cultures, the known alkaloids are reported as altogether absent or when present are in traces. Although secondary metabolites may be produced in large quantities by cultures, but the type of compound produced is often unexpected or even novel and specific compounds of the species might not be produced at all (Stockight *et al.*, 1981). Stockight *et al.* (1981) was able to screen 10 previously not reported alkaloids from cell cultures of *R. serpentina*. This does not necessarily imply that best known plant products or particular secondary metabolites can only be produced by organised cell system but suggests that cells growing *in vitro* under arbitrary conditions do not always produce a characteristic secondary metabolite.

However, now techniques are available to modify and improve the biosynthetic potential of cultured plant cells. In some plant species, cultures contain

higher contents of secondary metabolites than the intact plants (Khanna and Jain, 1973; Khanna and Manot, 1976; Khanna *et al.*, 1978). Recently a rather new technique using enzyme systems for regulating biosynthetic potentials in *Rauwolfia* cultures is given by Stockigt (1984) and Pfitzner *et al.* (1984). With these techniques and improvements, plant tissue and cell cultures systems could play an important role in the manufacture of biological compounds of therapeutic value.

In the present investigation various *in vitro* aspects of tissue culture have been tried with different explants of *R. serpentina* for the –

1. Determination of the best explants capable of quick multiplication,
2. Selection of a suitable nutrient medium in which regeneration is maximum.
3. Finding out the best possible way of transplantation of plantlets finally from the test tubes into the soil. and
4. Comparison of the amount of alkaloid in the field grown and *in vitro* grown plants and also the effect different growth factors supplemented in the media for alkaloid production in the *in vitro* grown plants.

MATERIALS AND METHODS

MATERIALS

The following types of explants viz., shoots, nodal segments, internodes, axillary buds and leaves were collected from field grown *R. serpentina* plants and were used for culture. In the subsequent experiments, *in vitro* grown clones were used as the source material for the present study.

METHODS

The methods involved in this investigation can be described under the following heads:

1. Preparation of MS culture media
2. Preparation of surface sterilizing solution
3. Culture techniques
4. Transplantation
5. Data collection
6. Estimation of reserpine

1. Preparation of MS Culture Media

The first step in the preparation of the MS medium (Murashige and Skoog, 1962) was the preparation of the stock solutions. Different constituents of different culture media formulations were prepared into stock as macronutrients, micronutrients, organic components and growth regulators separately for ready use during the preparation of the culture media.

A. Stock Solution of Macronutrients (Solution A)

This was made in such a way that its strength was ten times the final strength of the medium in 1 litre distilled water. For this purpose ten times the weight of different salts required for 1 litre of medium were weighted accurately and added one after another to 1 litre volumetric flask with 750 ml distilled water. Final volume of the solution was made up to 1 litre by adding sufficient amount of distilled water.

Special care was taken during dissolving $CaCl_2$. The solution was then filtered through Watman No.1 filter paper and was poured into a clean plastic container and stored in a refrigerator at 4°C for several weeks.

B. Preparation of Stock Solution of Micronutrients (Solution B).

In this case two separate stock solutions were prepared as follows:

i) Stock solution of $FeSO_4$ and Na_2-EDTA (Solution B,).

This was made ten times the final strength of F_2SO_4, $7H_2O$ and Na_2-EDTA in 100 ml distilled water and was chelated by heating and constant stirring into a water bath. Finally the stock solution was filtered and stored in a refrigerator at 4°C.

ii) Stock solution of the rest of the micronutrients (Solution B_2).

This was made ten times the final strength of all the micronutrients of the medium except $FeSO_4$, $7H_2O$ and Na_2-EDTA. All the components were weighed (except $CaCl_2$) separately and dissolved in 400 ml distilled water. $CaCl_2$ was dissolved separately and added to the solution. Finally the volume of the solution was adjusted up to 500 ml and after filtering was stored in a refrigerator.

C. Stock Solution of Organic Compounds (Solution C)

Stock solutions of each of the recommended ingredients of organic compounds were made separately and for this ten times of each of the required ingredient was weighed accurately and taken in 100 ml volumetric flask with 50 ml distilled water. It was swirled to dissolve and the final volume was made up to the mark by further addition of distilled water. Then these solutions were filtered and poured into plastic containers and after labeling were stored in a refrigerator at 4°C.

D. Stock Solution of Phytohormones (Solution D)

Stock solution of different phytohormones was prepared separately. The phytohormones were dissolved in the appropriate solvent as shown against each of them:

Hormone (Solute)	Solvent
IAA	0.1N KOH
IBA	0.1N KOH
2,4–D	70% Ethanol
NAA	0.1N KOH
BA	0.1N KOH (HCl)
Kn	0.1N HCl
GA_3	0.1N HCl

To prepare any of the hormonal stock solution, 10 mg of solid hormone was placed on a clean test tube and then dissolved in 1 ml of respective solvent. Final volume of the solution was made up to 100 ml by adding distilled water. The solution was then filtered and poured into a glass container and after labeling was stored at 4°C.

E. Stock Solution of Vitamins and Amino acids (Solution.E)

Pyridoxin HCl (Vit. B_6), thiamine HCl (Vit. B_1), nicotinic acid (niacin), glycine and myoinositol (inositol) were used.

Ten times each of the above mentioned vitamins and amino acids were dissolved separately in distilled water. They were then mixed and the volume was then made up to 100 ml by additional distilled water. The stock solution was stored in refrigerator.

Preparation of Culture Medium

For the preparation of 1 litre of any of the above media, the following steps were followed:

i) 100ml stock solution of macronutrients, 10ml from each of the micronutrients stock solution and 10ml from each vitamin stock solution were added to 750ml of distilled water and mixed well.

ii) Different required concentrations of hormonal supplements were added to this solution either individually or in combination and were mixed thoroughly. Since each 100 ml hormonal stock solution contained 10 mg hormonal salts, so addition of 10 ml of any hormonal stock solution to make 1 litre of medium resulted in 1.0 mg/ l concentration. Similarly, 1, 2 and 5 ml may contribute to 0.1, 0.2 and 0.5 mg/ l concentration.

iii) The whole mixture was then made up to 1 litre with further addition of distilled water.

iv) pH of the medium was adjusted to 5.7 by using an analogue pH meter with the help of 0.1N HCl, 0.1N KOH, 0.1N NaOH and 0.1N $Ca(OH)_2$ whichever was necessary.

v) 30 g of sucrose for MS basal medium was dissolved in 1000 ml of mixed components.

vi) To prepare solid medium, 7.0g bacteriological agar was added to the solution and the whole mixture was gently heated in a water bath with continuous stirring till complete dissolution of agar.

vii) Fixed volume of hot medium was taken into culture vessels namely test tubes and conical flasks. The culture vessels were plugged with non- absorbent cotton and marked with different codes with the help of a glass marker to indicate specific hormonal supplements.

viii) The culture vessels were then autoclaved at 121°C for 20 minutes at 1.1 kg/cm pressure. In case of test tubes, media were allowed to cool either as slanting or vertically, after sterilization.

2. Preparation of Surface Sterilizing Solution

Mercuric chloride ($HgCl_2$) solution at various concentrations, generally 0.1%, was used for surface sterilization of plant materials. To prepare 0.1% solution, 1g of $HgCl_2$ was taken in a 1 litre bottle and dissolved in 1000ml sterilized distilled water. Freshly prepared $HgCl_2$ was always used. Bleach solution at various concentrations, generally 10%, was used.

3. Culture Techniques

The following techniques were employed in the present investigation for induction and maintenance of callus induction, shoot induction, root induction as well as regeneration of complete plantlet:

a) Surface Sterilization of Plant Materials

Surface sterilization was carried out in the aseptic condition in a laminar airflow cabinet. The field grown plant materials, namely shoot tips, nodal segments, leaves and internodal segments were taken into a sterile conical flask and suspended in different concentrations of $HgCl_2$ for different periods of 5, 10, 15, 25, and 30 minutes to ensure contamination free culture. Then the plant materials were washed 3-4 times with sterile distilled water.

b) Preparation of Explants and Inoculation

Explants were laid on the sterile petri dish using sterile forceps. During this action hands were made sterile as far as possible with absolute alcohol to avoid contamination.

i) Shoot tip: Shoot tips of approximately 1 to 2 cm in length were cut from the sterilized shoot of mature plants and then inoculated into the culture tubes having different concentrations and combinations of hormones.

ii) Nodal segments: In the same way mentioned above internodal segments approximately 1 cm in length were cut from the shots and were inoculated into culture tubes.

iii) Internodal segments: In the same way mentioned above internodal segments approximately 1 cm in length were cut from the shoots and were inoculated into culture tubes.

iv)Leaves: In the same way mentioned above tender leaves were cut and placed into the culture tubes.

c) Incubation

The culture tubes containing inocula were incubated to light in the growth chamber. The temperature of the growth chamber was 27 + 1°C and the photoperiod were 16h per day with photointensity of 2000 lux.

d) Subculture Techniques

The following *in vitro* culture techniques were employed during the course of culturing of different explants.

i) Subculture maintenance: Proliferated multiple shoots were rescued very carefully in the aseptic conditions and divided into clusters of 2-3 shoots using a sterile sharp scalpel. Then they were transferred to same or different media for further response.

ii) Root induction: Proliferated shoots of 2-3 cm in length were rescued aseptically from the culture vessels and cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

iii)Callus induction: The *in vitro* grown shoots of 3-5 cm in length were rescued aseptically from the culture vessels and internodes as well as leaves were cultured of freshly prepared medium containing different concentrations and combinations of hormonal supplements for callus induction.

4. Transplantation

After sufficient growth of shoot and root systems, the plantlets were considered ready to transfer in soil. The plantlets grown inside the test tubes/flasks were brought out of the control environment of growth chamber and were kept in the room temperature for 5-7 days to bring them in contact of normal temperature. The plantlets were then rescued very carefully from the culture vessels. Agar attached to the root system was gently washed out under running tap water. Then the plantlets were transplanted to small polythene bags containing garden soil and compost in the ratio of 2 : 1. The soil substance was treated with 0.1% Agrosan (fungicide) solution. Immediately after transplantation, the plantlets along with the polythene bags were covered with a large moist polythene bag to prevent desiccation. To obtain higher humidity around the plantlets, all the bags were checked up and the interiors of the polythene bags were sprayed with water at every 24 hours. The polythene bags were gradually perforated to expose the plantlets to the outer environment and subsequently removed after 10 days. By this time new leaves emerged out and the regenerates became established in the soil being complete plantlets. They were then transferred to garden soil.

5. Data Collection

During collection of data in different experiments following parameters were considered:

i) Average number of shoots and length of the longest shoot: Number of multiple shoots per explant was counted after 15, 30 and 45 days from the date of inoculation.

ii) Shoot height was recorded separately with meter scale. Average height of the longest shoot was calculated.

iii) Average number of roots per shoot and length of the longest shoot: Number of main roots were counted after 20, 40 and 60 days from the date of inoculation.

iv) Rate of callus forming explant: Cultured explants, which showed callus formation, were recorded after 60 days of inoculation. The degree of callus formation was recorded as follows:

– = No callus, + = Trace callus development (1 – 30%), + + = Moderate callus development (31 – 50%) and + + + = Massive callus formation (51 – 100%).

v) After 45 days of culture each explant with proliferating callus was taken out from the culture media and washed in the running tap water. Then the callus was placed under a fan for 2–3 hours to remove water and weighed carefully with an electronic balance. Weight of the callus was taken in g.

vi) After fresh weight, the calluses were placed on petri dishes and kept in an oven for 3–4 days at 55°C for drying.

6. **Procedure for Estimation of Reserpine**

In vitro grown plantlets and calli were taken out from the culture media and washed in the running tap water. Then the plant parts (shoot, leaf, root and base callus) were separated and were placed under a fan for 2-3 hours to remove water. Fresh weight of each of the plant parts as well as calli were taken. Next, plant parts and calli were placed separately on petri dishes and kept in an oven for 3-4 days at

55°C for drying. Dry weight of the plant parts and calli was also recorded. Then the dried materials were made fine powder by a mortar and pestle and stored for reserpine estimation as in Chapter 2. In case of field grown plants the same procedure was followed.

RESULTS

SHOOT MULTIPLICATION IN DIFFERENT COMBINATIONS AND CONCENTRATIONS OF PHYTOHORMONES

In vitro multiple shoot formation is an important aspect of tissue culture experimentation. This part includes shoot multiplication with various cytokinins, combination of cytokinins and auxin and combination of cytokinins and gebberellic acid (GA_3) in MS medium. The first experiment was set up with field grown nodal explants having one axillary bud. The subsequent experiments were carried out using nodal segments and shoot apices of *in vitro* grown shoots. Data collected on percentage of shoot formation, average number of shoot per explant, average shoot length, fresh and dry weights of shoots were analyzed.

Effect of Different Concentrations of BA on Shoot Multiplication from Field Grown Nodal Segment

Nodal explants each having one axillary bud were collected from the field grown two years old *R. serpentina* plants for *in vitro* shoot formation. Detailed

procedures have been described in Materials and Methods section of Chapter 6. Here 6 different levels (1 to 6 mg/l) of BA were used.

Results obtained for different parameters are shown in Table 20.

Percentages of cultures responded to multiple shoot formation on different levels of BA varied considerably. None of the cultures responded when BA level was used below 1.0 mg/l. The lowest percentage (7%) of multiple shooting was recorded in 1.0 mg/l BA. On the other hand, the highest percentage (42.6%) was recorded in 4.0 mg/l BA and gradually decreased thereafter.

Table 20 : Effect of different concentrations of BA on shoot multiplication from field-grown nodal segment of *R. serpentina*.

Growth regul-ators (mg/l)	% of shoot formati on	Average no. of shoots/explant after days			Average length (cm) of shoot after days			Average wt. of shoots/explant (g) after 45 days		Base Callu sing
		15	30	45	15	30	45	Fresh wt.	Dry wt.	
1.0	7.0	1.0	1.0	1.0	0.5	0.9	1.7	1.09	0.17	-
2.0	15.0	1.5	2.0	2.1	0.5	1.0	1.8	1.74	0.18	-
3.0	22.4	1.5	2.2	2.3	0.6	1.2	2.1	1.93	0.20	+
4.0	42.6	1.7	2.3	2.6	0.6	1.2	1.9	2.00	0.22	++
5.0	32.3	1.9	1.8	2.4	0.8	1.5	1.7	1.82	0.17	++
6.0	27.8	1.6	1.6	2.0	0.6	1.2	1.5	1.68	0.16	+
$LSD_{5\%}$	5.2	NS	0.5	0.6	NS	NS	0.4	0.24	NS	

Average number of shoots was recorded at 15, 30 and 45 days of culture. Average number of shoots per culture was always less than 2 at 15 days of culture. Highest average number of shoots was achieved at 45 days of culture in 4.0 mg/l BA (Plate I, A). The lowest average number of shoot was recorded in 1.0 mg/l BA.

Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 15, 30 and 45 days of culture. At 15 days of culture, mean length of shoots was always less than 1 cm in all BA levels used. However, higher average length was recorded at 45 days of culture in 3 mg/l BA. On the other hand, the lowest average length was noted in 6 mg/l BA.

Fresh weight of shoots per culture was recorded at 45 days of culture. The highest fresh weight (2.00 g/culture) was recorded in the media having 4.0 g/l BA. The lowest fresh weight (1.09 g/culture) was noted in 1.0 mg/l BA.

After taking fresh weight, shoots were dried at 55°C for 5 days in a temperature-controlled dryer and dry weights were taken (Table 20). The highest dry weight of 0.22 g/culture was recorded in 4.0 mg/l BA.

Moderate base callusing was observed in 4.0 mg/l and 5.0 mg/l BA. Trace base callusing was noted in 3.0 and 6.0 mg/l BA. No base callusing was noted in the rest of the media used.

Effect of Different Concentrations and Combinations of BA with NAA and GA_3 on Shoot Multiplication from *in vitro* Grown Nodal Segment

In this experiment, effects of different concentrations of BA singly as well as in combination with NAA and GA_3 was studied.

Effect of different concentrations of BA

Ten different concentrations of BA used were 0.1, 02, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0. Results are shown in Table 21. Percentage of cultures induced multiple shoots dependent on the concentrations of BA used in MS medium. The range of percentage of culture responded multiple shooting was 7.2% in 0.5 mg/l to 57.0% in 3.0 mg/l BA. MS medium containing 0.1, 0.2 and 7.0 mg/l BA failed to induce any multiple shoots.

Table 21 : Effect of different concentrations of BA on shoot multiplication from *in vitro* grown nodal segments of *R. serpentina.*

Growth regul-ators (mg/l)	% of shoot forma-tion	Average no. of shoots/explant after days			Average shoot length (cm) after days			Average wt of shoots/explant (g) after 45 days		Base Callusing
		15	30	45	15	30	45	Fresh Wt.	Dry Wt.	
0.5	7.2	1.0	1.0	2.0	0.7	1.7	2.6	2.53	0.31	-
1.0	12.5	1.8	2.3	2.7	0.8	2.0	2.8	2.54	0.32	+
2.0	48.5	2.6	2.9	3.1	0.9	2.1	3.5	2.58	0.39	+ +
3.0	57.0	2.3	2.8	3.6	0.8	1.8	3.7	2.67	0.47	+++
4.0	45.2	2.2	2.6	2.8	0.7	1.6	2.7	2.31	0.31	++
5.0	40.6	2.0	2.2	2.3	0.5	1.4	2.3	2.01	0.27	+
6.0	31.6	1.1	1.3	1.6	0.4	1.2	2.1	2.10	0.19	+
$LSD_{5\%}$	7.5	0.4	0.6	0.6	NS	0.3	0.6	0.15	0.12	

The average number of shoots per explant was 1.0 to 2.6 at 15 days of culture. There was a gradual increase in the number of shoots per explant up to 45 days. The highest number of shoots per explant was recorded in 3.0 mg/l BA which was 3.6 shoots per culture (Plate-1, B). MS medium which contained more than 3.0 mg/l BA did not increase the number of shoots per culture but decreased from 3.6 shoots per culture in 3.0 mg/l to 1.6 shoots per culture in 6.0 mg/l BA.

Average length of the longest shoot was found to be affected by different concentrations of BA used in the MS media. The length of the longest shoot was always less than 1 cm at 15 days of culture in all the concentrations of BA. The length of the shoot gradually increased from 15 days to 45 days of culture. The highest length of the shoot was 3.7 cm in 3.0 mg/l BA and the lowest length was recorded in 6.0 mg/l BA which was 2.1 cm.

The highest fresh weight of shoot per culture was recorded in 3.0 mg/l BA and the lowest in 5.0 mg/l BA. The dry weight of shoots was also influenced by the various concentrations of BA used. The highest dry weight per culture (0.47 g) in 3.0 mg/l BA. Compared to 0.5 to 3.0 mg/l BA, dry weight of shoots was lower in 5.0 and 6.0 mg/l BA.

Base callusing was recorded in some culture media. The highest amount of base callusing (+ + +) was recorded in culture where 3.0 mg/l BA was used. Base callusing was moderate in both 2.0 and 4.0 mg/l BA. No base callusing was recorded in media having 0.5 mg/l BA.

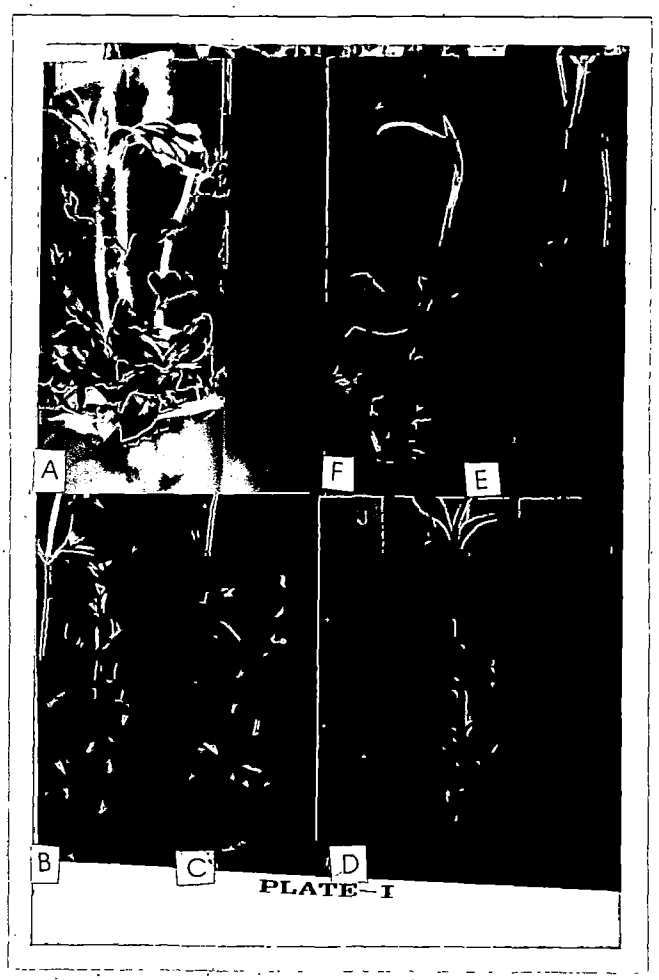


Plate I

A. Primary establishment in aseptic condition in medium having MS + 4.0 mg/l BA from field grown nodal explant,

B. Multiple shoot formation in medium having MS + 3.0 mg/l BA from *in vitro* grown nodal segment.

C. Shoot formation in medium having MS + 2.0 mg/l BA + 0.2 mg/l NAA from *in vitro* grown nodal segment.

D. Multiple shoot formation in medium having MS + 3.0 mg/l Kn + 0.5 mg/l NAA.

E. Multiple shoot formation in medium having MS + 2.0 mg/l BA + 0.1 mg/l GA_3..

F. Multiple shoot formation in medium having MS + 2.0 mg/l Kn + 0.5 mg/l GA_3.

Effect of different concentrations of BA and NAA.

In this experiment five concentrations of BA (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l) were used with each of the 4 different concentrations of NAA (0.1, 0.5, 1.0 and 2.0 mg/l) were supplemented with MS media.

Results are presented in Table 22.

There was no response in 0.1 mg/l BA with any of the 4 concentrations of NAA.

Percentage of cultures responded to multiple shooting ranged from 21.5% to 82%, it depended on the concentrations of BA and NAA used in the media. The highest percentage of cultures responded to multiple shoots in 2.0 mg/l BA with all the 4 concentrations of NAA. It ranged from 54.6% in 2.0 mg/l BA +1.0 mg/l NAA to 82% in 2.0 mg/l BA + 0.2 mg/l NAA. The percentage of response in 1.0 mg/l BA with all the 5 concentrations of NAA ranged from 21.5 to 48.5.

Average number of shoots per culture was highest in 2.0 mg/l BA + 0.2 mg/l NAA which were 3.0,3.3 and 3.8 respectively at 15, 30, and 45 days of culture (Place – I, C). More than 3 shoots per culture were recorded in 2.0 mg/l BA with 0.1 and 0.5 mg/l NAA. The lowest number of shoots per culture was recorded in 0.5 mg/l BA with all the 4 concentrations of NAA.

Average length of longest shoots was also influenced by the different concentrations of BA used. The highest average length of the longest shoot was 4.6 cm in 2.0 mg/l BA + 0.2 mg/l NAA. Average length of the longest shoots was low in 0. 5 and 3.0 mg/l BA with all the concentrations of NAA used.

Fresh weight of shoots per culture was recorded after 45 days of culture and was high in 1.0 and 2.0 mg/l BA with all the concentrations of NAA used. The highest fresh weight was recorded to be 3.86 g per culture in 2.0 mg/l BA + 0.2 mg/l NAA. Fresh weight of less than 3.0 g per culture was recorded in 0.5 and 5 mg/l BA.

Table 22 : Effect of different concentrations and combinations of BA with NAA and GA_3 on shoot multiplication from *in vitro* grown nodal segment of *R. serpentina*.

Growth regulators (mg/l)	% of shoot formation	Average no. of shoots/explant after days			Average shoot length (cm). after days			Average wt.of shoots/explant (g) after 45 days		Base Callusing
		15	30	45	15	30	45	Fresh wt.	Dry wt.	
BA+NAA										
0.5+0.1	21.5	1.6	1.7	2.1	0.9	1.9	2.8	2.87	0.35	
0.5+0.2	28.7	2.2	2.6	2.8	1.1	2.0	2.9	2.87	0.34	-
0.5+0.5	42.3	2.1	2.3	2.4	1.4	1.9	3.1	2.98	0.36	-
0.5+1.0	32.8	1.4	1.5	1.5	1.3	1.8	2.7	2.96	0.35	-
1.0+0.1	47.27	2.2	2.7	3.0	1.4	2.6	3.4	3.37	0.44	++
1.0+0.2	48.5	2.4	2.8	2.9	1.6	2.8	3.6	3.35	0.43	+
1.0+0.5	34.6	2.0	2.2	2.5	1.5	2.6	3.3	3.51	0.66	+
1.0+1.0	21.5	1.5	1.6	2.0	1.4	3.0	3.8	3.44	0.62	+
2.0+0.1	68.2	2.8	2.9	3.1	1.7	3.4	4.2	3.71	0.77	+

Contd.

Table 22 (Contd.)

2.0+0.2	82.0	3.0	3.3	3.8	2.1	3.8	4.6	3.86	0.81	+++
2.0+0.5	68.5	2.9	3.2	3.4	2.1	3.2	3.9	3.52	0.66	++
2.0+1.0	54.6	2.3	2.4	2.6	1.8	2.6	3.2	3.37	0.42	+
3.0+0.1	40.0	2.1	2.2	2.3	1.8	2.3	2.8	2.83	0.27	-
3.0+0.2	35.2	2.0	2.0	2.2	1.8	2.6	2.4	2.63	0.25	-
3.0+0.5	33.1	1.7	2.0	2.1	1.7	2.0	1.9	1.92	0.18	-
3.0+1.0	32.0	1.6	1.7	2.1	1.7	1.9	1.9	1.81	0.17	-
$LSD_{5\%}$	12.2	0.3	0.5	0.6	NS	NS	0.6	0.25	0.11	
BA+ GA_3										
0.5+0.1	4.0	1.0	1.0	2.0	0.9	1.8	2.7	3.05	0.41	-
0.5+0.5	6.2	1.0	1.8	2.2	1.0	1.9	2.8	2.98	0.36	-
0.5+1.0	14.0	1.0	1.6	2.1	1.0	1.9	2.6	2.96	0.44	-
0.5+2.0	13.5	1.0	1.6	2.2	1.0	1.9	2.4	2.67	0.28	-
0.5+3.0	7.5	1.0	1.0	1.0	0.8	2.0	2.2	2.17	0.24	-
1.0+0.1	18.0	2.1	2.4	2.7	0.9	2.0	2.9	3.14	0.43	-
1.0+0.5	28.5	1.6	1.8	1.9	0.9	2.0	3.1	3.15	0.41	-
1.0+1.0	36.0	1.5	1.7	1.9	1.3	2.0	3.6	3.20	0.44	-
1.0+2.0	37.8	1.5	1.5	1.7	1.4	2.1	2.8	3.12	0.40	-

Contd.

Table 22 (Contd.)

1.0+3.0	18.4	1.0	1.6	1.6	1.0	1.8	2.6	2.99	0.38	-
2.0+0.1	62.0	2.4	2.8	3.5	1.0	2.6	4.0	3.67	0.51	-
2.0+0.5	52.8	2.3	2.7	3.2	0.9	2.4	3.5	3.42	0.43	-
2.0+1.0	44,3	2.0	2.1	2.9	0.8	2.3	3.2	3.28	0.43	-
2.0+2.0	32.6	1.5	1.7	2.0	0.7	2.0	3.1	3.11	0.36	-
2.0+3.0	24.0	1.0	1.3	1.6	0.6	1.9	1.8	1.85	0.20	-
3.0+0.1	48.0	2.5	2.5	2.6	0.7	1.4	2.5	2.98	0.40	-
3.0+0.5	32.0	2.3	2.3	2.6	0.7	1.4	2.4	2.82	0.35	-
3.0+1.0	33.5	2.0	2.4	2.5	0.5	1.4	2.0	2.35	0.29	-
3.0+2.0	17.2	2.0	2.4	2.4	0.6	1.3	1.6	2.24	0.26	-
3.0+3.0	12.3	1.6	2.1	2.4	0.3	1.1	1.3	2.10	0.22	-
4.0+0.1	32.0	2.3	2.3	2.6	0.5	1.4	2.2	2.01	0.20	-
4.0+0.5	23.0	2.1	2.2	2.4	0.4	1.2	1.9	1.81	0.18	-
4.0+1.0	18.2	1.0	2.1	2.1	0.4	1.0	1.8	1.80	0.18	-
4.0+2.0	11.4	1.0	1.0	1.1	0.3	0.9	1.4	1.62	0.15	-
4.0+3.0	-	-	-	-	-	-	-	-	-	-
$LSD_{5\%}$	14.3	0.6	0.5	0.6	0.4	NS	NS	0.15	0.10	

Dry weight of shoots per culture was highly influenced by the various concentrations of BA and NAA used. The lowest dry weight of 0.17 g per culture was recorded in 3.0 mg/l BA + 1.0 mg/l NAA and the highest dry weight was recorded in 2.0 mg/l BA + 0.2 mg/l NAA which was 0.81 g per culture.

Base callusing was noted in all the NAA concentrations where 1.0, 2.0 and 3.0 mg/l BA were used. The highest concentration of base callusing was recorded in 2.0 mg/l BA + 0.2 mg/l NAA. Moderate base callusing was noted in 1.0 mg/l BA + 0.1 mg/l NAA, 2.0 mg/l BA + 0.01 mg/l NAA, 2.0 mg/l BA + 0.2 mg/l NAA and 2.0 mg/l BA + 0.5 mg/l NAA. In the rest of the media, a little amount of base callusing was noted. All the 4 concentrations of NAA with 0.5 and 3.0 mg/l BA did not induce any base callus in any of the culture media.

Effect of different concentrations of BA and GA_3

MS media were supplemented with 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l BA and each of 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l GA_3.

There was no response observed in 4.0 mg/l BA with 3.0 mg/l GA_3. Percentage of cultures responded to produce multiple shoots ranged from 4% to 62% and was influenced by different concentrations and ratio of BA and GA_3 used in the media (Table 22). The highest percentages of culture (62%) responded in 2.0 mg/l BA + 0.1 mg/l GA_3. The lowest percentage of response (4%) was recorded in 0.5 mg/l BA with 0.1 mg/l GA_3.

Average number of shoots per culture was highest in 2.0 mg/l BA with all the concentrations of GA_3 (0.1, 0.5, 1.0 and 2.0 mg/l). The maximum number of shoots per culture was recorded in 2.0 mg/l BA + 0.1 mg/l GA_3 (Plate – I, E). More than 2

shoots per culture were noted in 2.0 mg/l BA and 3.0 mg/l BA with all the concentrations of GA_3 used. The average number of shoots per culture was found to be lowest in 4.0 mg/l BA with most of the GA_3 concentrations used.

Average length of longest shoots per culture was also influenced by the various concentrations of BA used. The highest average length of shoots was 3.5 cm in 2.0 mg/l BA + 0.1 mg/l GA_3 whereas lower length of the longest shoot was found in 3.0 mg/l and 4.0 mg/l BA with all the levels of GA_3 used.

Fresh weight of shoots per culture was weighed after 45 days of culture. The highest fresh weight was recorded in the media supplemented with 2.0 and 3.0 mg/l BA with all the 5 concentrations of GA_3 except in 3.0 mg/l GA_3. Fresh weight was less than 2.0-g/ culture in 4.0 mg/l BA with all the concentrations of GA_3 besides 0.5 mg/l GA_3.

Dry weight of shoots per culture was greatly influenced by the different concentrations of BA used. The highest dry weight was noted in 2.0 mg/l BA + 0.1 mg/l GA_3 (0.51g) and the lowest dry weight was 0.15 g/culture and recorded in 4.0 mg/l BA + 2.0 mg/l GA_3.

No base callusing was found in any of the combinations used.

Effect of Different Concentrations and Combinations of Kn With NAA And GA_3 on Shoot Multiplication from *in vitro* Grown Nodal Segment

Effect of different concentrations and combinations of Kn and NAA.

The experiment was set up to observe the effect of combined action of Kn and NAA on multiple shoot formation from nodal segment. The concentrations of Kn were 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l which were used with any one of 4 concentrations of NAA (0.1, 0.5, 1.0 and 2.0 mg/l).

Results obtained on percentage of cultures responded, shoot number per explant, shoot length and fresh and dry weights of shoots per culture are tabulated in Table 23.

No response of culture was recorded in 0.1 mg/l Kn with any of the 5 concentrations of NAA. Percentage of cultures responded to multiple shooting ranged from 10 to 56, it depended on the concentration and ratio of Kn and NAA used in the media. Higher percentage of cultures responded was noted in 3.0 mg/l Kn with all the 4 concentrations of NAA. It ranged from 40% (at 3.0 mg/l Kn + 0.1 mg/l NAA) + 0.56% (at 3.0 mg/l Kn + 0.6 mg/l NAA).

Average number of shoots per culture was highest in 3.0 mg/l Kn + 0.5 mg/l NAA, which were 2.3, 2.7 and 3.0 at 15, 30 and 45 days of culture, respectively. The lowest number of shoots per culture was recorded in 4.0 mg/l Kn with 1.0 and 2.0 mg/l NAA concentrations (Plate – I, D).

The highest average length of the longest shoot was 2.4 cm in 2.0 mg/l Kn + 1.0 mg/l NAA. The lowest average length of shoot was found in 4.0 mg/l Kn + 2.0 mg/l NAA.

Table 23 : Effect of different concentrations and combinations of Kn with NAA and GA_3 on shoot multiplication from *in vitro* grown nodal segment of *R. serpentina.*

Growth regul-ators (mg/l)	% of shoot forma-tion	Average no. of shoots/explant after days			Average shoot length (cm). after days			Average wt. of Shoots/ explant (g) after 45 days		Base Callu sing
		15	30	45	15	30	45	Fresh wt.	Dry wt.	
Kn+NAA										
0.5+0.1	10.0	1.0	2.0	2.0	0.6	1.3	2.5	1.81	0.21	-
0.5+0.5	18.5	1.0	1.5	2.0	0.5	1.2	2.3	1.82	0.20	-
0.5+1.0	26.0	1.6	2.0	2.3	0.7	1.1	2.1	1.93	0.22	-
0.5+2.0	26.5	1.7	2.0	2.3	0.4	1.3	2.2	1.90	0.21	-
1.0+0.1	18.3	1.5	2.0	2.5	0.7	1.3	2.1	1.97	0.24	-
1.0+0.5	26.0	1.7	2.0	2.3	0.6	1.5	2.3	1.95	0.24	-
1.0+1.0	32.0	1.7	2.2	2.5	0.6	1.4	2.4	1.96	0.24	-
1.0+2.0	26.6	2.0	2.3	2.3	0.7	1.3	2.2	1.93	0.23	-
2.0+0.1	32.8	1.6	2.0	2.2	0.5	1.1	2.0	1.89	0.20	-
2.0+0.5	34.5	1.6	2.2	2.5	0.4	1.0	2.1	1.92	0.21	-
2.0+1.0	40.6	2.0	2.4	2.6	0.6	1.1	2.4	1.87	0.20	-
2.0+2.0	42.0	2.2	2.4	2.4	0.5	0.9	1.9	1.81	0.18	-
3.0+0.1	40.0	2.2	2.6	2.8	0.4	0.7	1.9	1.98	0.26	-
3.0+0.5	56.0	2.3	2.7	3.0	0.5	0.9	2.0	2.01	0.29	-
3.0+1.0	48.2	2.2	2.4	2.6	0.3	0.8	2.1	1.96	0.25	

Contd.

Table 23 (Contd.)

3.0+2.0	55.0	1.9	2.0	2.1	0.5	0.7	2.2	1.83	0.21	-
4.0+0.1	40.0	1.5	1.7	2.0	0.5	0.8	1.9	1.82	0.20	-
4.0+0.5	40.5	1.5	2.0	2.0	0.5	0.9	2.1	1.81	0.19	-
4.0+1.0	30.0	1.0	1.5	1.5	0.4	1.0	1.8	1.78	0.18	-
4.0+2.0	16.5	1.0	1.0	1.0	0.3	0.7	1.7	1.72	0.16	-
$LSD_{5\%}$	18.5	0.5	0.6	0.4	0.4	0.5	0.3	0.20	0.11	
$Kn+GA_3$										
0.5+0.1	7.0	1.0	1.0	1.0	0.5	0.9	1.9	1.86	0.17	-
0.5+0.5	7.0	1.0	1.0	1.0	0.6	0.9	1.8	1.87	0.17	-
0.5+1.0	13.3	1.5	1.5	2.0	0.6	1.0	2.2	1.92	0.19	-
0.5+2.0	13.3	1.5	1.5	1.5	0.7	1.0	2.0	1.93	0.18	-
0.5+3.0	7.0	1.0	1.0	1.5	0.4	0.7	1.3	1.60	0.14	-
1.0+0.1	13.3	1.0	1.5	1.5	0.6	1.1	2.4	1.98	0.22	-
1.0+0.5	26.6	1.7	2.0	2.2	0.8	1.4	2.8	2.12	0.20	-
1.0+1.0	33.3	1.8	2.0	2.3	0.9	1.5	2.7	2.14	0.21	-
1.0+2.0	33.3	1.8	2.0	1.5	0.7	1.4	2.3	1.88	0.18	-
1.0+3.0	7.0	1.0	1.0	1.5	0.4	1.6	1.9	1.79	0.17	-
2.0+0.1	33.3	1.4	1.8	1.8	0.7	1.3	2.0	1.90	0.18	-
2.0+0.5	40.0	2.0	2.3	2.5	0.8	2.1	3.0	2.24	0.23	-

Contd.

2.0+1.0	26.6	1.2	1.5	1.7	0.5	1.1	1.9	1.90	0.19	-
2.0+2.0	26.6	1.0	1.5	1.7	0.5	0.8	1.0	0.99	0.10	-
2.0+3.0	13.3	1.0	1.0	1.0	0.5	0.7	1.0	.96	0.09	-
3.0+0.1	33.3	1.2	1.4	1.8	0.6	1.0	1.9	1.95	0.19	-
3.0+0.5	26.6	1.0	1.5	1.5	0.6	1.0	1.9	1.96	0.18	-
3.0+1.0	20.0	1.0	1.0	1.5	0.4	0.7	1.5	1.72	0.16	-
3.0+2.0	13.0	1.0	1.0	1.0	0.4	0.7	1.3	1.65	0.15	-
3.0+3.0	6.0	1.0	1.0	1.0	0.3	0.5	1.0	1.05	0.10	-
4.0+0.1	26.6	1.0	1.5	1.5	0.5	0.9	1.5	1.68	0.17	-
4.0+0.5	26.0	1.0	1.0	1.5	0.5	0.8	1.3	1.61	0.12	-
4.0+1.0	13.3	1.0	1.0	1.5	0.3	0.7	1.2	1.58	0.11	-
4.0+2.0	6.0	1.0	1.0	1.0	0.3	0.5	0.9	1.01	0.09	-
4.0+3.0	-	-	-	-	-	-	-	-	-	-
$LSD_{5\%}$	8.2	NS	NS	0.3	NS	0.5	0.3	0.15	0.08	

Fresh weight of shoots per culture was obtained after 45 days of culture. The highest fresh weight was recorded as 2.01 g/culture in 3.0 mg/l Kn + 0.5 mg/l NAA.

Dry weight of shoots per culture was highly influenced by the various concentrations of Kn and NAA. The highest dry weight of 0.29 g/culture was recorded in 3.0 mg/l Kn + 0.5 mg/l NAA and the lowest dry weight of 0.16 g/culture was noted in 4.0 mg/l Kn + 2.0 mg/l NAA.

No base callusing was observed in any of the combinations of Kn and NAA used.

Effect of Different Concentrations and Combinations of Kn and GA_3.

This experiment was conducted to find out the effect of Kn and GA_3 on multiple shoot formation. Five different concentrations (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of Kn were used with 5 concentrations of GA_3 (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l).

The results are presented in Table 23. There was no response in 4.0 mg/l Kn + 3.0 mg/l GA_3. Percentage of cultures induced multiple shooting ranged from 7% to 40% depending on the concentrations of Kn and GA_3 used in the media (Table 23). The highest percentage of cultures induced multiple shooting in 2.0 mg/l Kn with 0.5 mg/l GA_3. The range of percentage was from 13% (in 2.0 mg/l Kn + 3.0 mg/l GA_3) to 40% (in 2.0 mg/l Kn + 0.5 mg/l GA_3). The percentage of cultures responded gradually decreased with the increase of Kn concentrations with GA_3.

Average number of shoots per culture was highest in 2.0 mg/l Kn + 0.5 mg/l GA_3 which were 2.0, 2.3 and 2.5 at 15, 30 and 45 days of culture (Plate – I, F). Only more than 2 shoots per culture was noted in 2.0 mg/l Kn with three concentrations of GA_3 (i.e. 0.5, 1.0 and 2.0 mg/l). The lowest number of shoots per culture was recorded in 4.0 mg/l Kn with all concentrations of GA_3. Average length of the longest shoot was also influenced by the concentrations of Kn and GA_3 used. The highest

average length of the longest shoot was 3.0 cm in 2.0 mg/l Kn + 0.5 mg/l GA_3. The lowest average length of the longest shoot was noted in 4.0 mg/l Kn with all concentrations of GA_3 used.

Fresh weight was influenced by the concentrations of Kn. The highest fresh weight (2.24 g/culture) was obtained in 2.0 mg/l Kn + 0.5 mg/l GA_3. The lowest fresh weight (1.01 g/culture) was recorded in 4.0 mg/l Kn + 2.0 mg/l GA_3.

Dry weight was also affected by the concentrations of Kn and GA_3. Dry weight was highest in 2.0 mg/l Kn + 0.5 mg/l GA_3 which was 0.23 g/l culture. The lowest dry weight of 0.09 g/culture was obtained in 4.0 mg/l Kn + 2.0 mg/l GA_3.

No base callusing was found in any of the culture media used.

Effect of Different Concentrations and Combinations of BA With NAA and GA_3 on Shoot Multiplication from *in vitro* Grown Shoot Tip

Here effect of different concentrations of BA singly as well as in combination with NAA and GA_3 was studied.

Effect of different concentrations of BA

Shoot tips were collected from *in vitro* grown plantlets and were cultured in MS medium supplemented with 9 different concentrations of BA i.e., 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/l.

Data obtained for percentage of cultures responded multiple shooting, average numbers of shoots per culture, average length of the shoots per culture and fresh and dry weights of shoots/explant are shown in Table 24.

The explants did not respond to multiple shooting when they were cultured on media supplemented with 0.1 to 0.5 mg/l BA. Only 13% of the cultures responded to induce multiple shooting in 1.0 mg/l BA that was found to be the least responded culture medium among all other media used. The highest percentages of cultures induced multiple shoots was recorded in media supplemented with 3.0 mg/l BA.

The highest number of shoots per culture was recorded at 45 days of culture (Plate–II, A). The number of shoots per culture was 2.6 on media where 4.0 mg/l BA was used. In other BA concentrations the shoots per culture was less than 2.6.

Average length of the longest shoots was less than 1 cm at 15 days of culture and it gradually increased to 3 cm at 45 days of culture. The range of shoot length was 1.9 cm to 3.0 cm at 45 days of culture.

Fresh weight of shoots was almost same in all the media composition except in 3.0 and 4.0 mg/l BA where greater fresh weight was obtained compared to other BA concentrations. Fresh weight of shoots per culture was 1.99 and 1.98 g respectively in cultures supplemented with 3.0 and 4.0 mg/l BA.

Table 24 : Effect of different concentrations of BA on shoot multiplication from *in vitro* grown shoot tip of *R. serpentina.*

Growth regulators (mg/l)	% of shoot formation	Average no. of shoots/explant after days			Average shoot length (cm). after days			Average wt. Of shoots/explant (g) after 45 days		Base Callusing
		15	30	45	15	30	45	Fresh Wt.	Dry Wt.	
BA										
1.0	13.0	2.1	2.2	2.5	0.8	1.9	2.7	1.72	0.17	+
2.0	40.0	2.1	2.3	2.5	1.0	1.9	3.0	1.78	0.18	++
3.0	53.0	2.2	2.3	2.5	1.1	1.8	2.8	1.99	0.20	+++
4.0	46.0	2.2	2.4	2.6	0.7	1.7	2.7	1.98	0.19	++
5.0	40.0	2.1	2.3	2.5	0.6	1.5	2.3	1.81	0.18	++
6.0	26.6	1.9	2.1	2.3	0.5	1.3	1.9	1.77	0.18	+
$LSD_{5\%}$	12.5	NS	NS	NS	NS	0.3	0.4	0.25	0.10	

Dry weight of shoots per culture was significantly higher in 3.0 and 4.0 mg/l BA supplemented media compared to other BA concentrations. Dry weight of shoots per culture was 0.20 and 0.19 g in 3.0 and 4.0 g/l BA supplemented media, respectively.

Moderate base callusing was recorded in cultures where 2.0, 4.0 and 5.0 mg/l BA were used. Trace callusing was also recorded in 3.0 and 6.0 mg/l BA.

Effect of different concentrations of BA and NAA

In this experiment effect of BA with NAA on shoot tip culture in respect of multiple shooting was studied. BA concentrations were 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l and each of the concentrations were used with 0.1, 0.2, 0.5, 1.0 and 2.0 mg/l NAA.

Results obtained on the percentage of cultures responded to multiple shooting, average number of shoots per culture and fresh and dry weights of shoots per culture and shown in Table 25.

None of the cultures responded when 0.1 mg/l BA was supplemented with all the 5 concentrations of NAA used in the culture media. In the other media composition, the explants responded and produced multiple shoots. The lowest percentage of cultures responded to multiple shooting was recorded in 0.5 mg/l BA with all the 5 concentrations of NAA which ranged from 6% to 13%.

Percentage of cultures was high when 2.0 and 3.0 mg/l BA were used in the culture media. Significant effect of NAA concentrations with BA was noted. The highest percentage of cultures responded to growth and multiple shooting was observed in lower concentrations of NAA with either 2.0 or 3.0 mg/l of BA. As high as 33% of cultures responded to growth and shooting was recorded in media supplemented with 2.0 mg/l BA + 0.2 mg/l NAA, 2.0 mg/l BA + 0.5 mg/l NAA and 3.0 mg/l BA + 0.5 mg/l NAA.

Table 25 Effect of different concentrations and combinations of BA with NAA and GA_3 on shoot multiplication from *in vitro* grown shoot tip of *R. serpentina*.

Growth regul-ators (mg/l)	% of shoot forma -tion	Average no. of shoots/explant after days			Average shoot length (cm). after days			Average wt. Of shoots/explant (g) after 45 days		Base Callusing
		15	30	45	15	30	45	Fresh wt.	Dry wt.	
BA+NAA										
0.5+0.1	6.0	1.0	1.0	1.0	0.6	1.3	2.5	2.30	0.24	+
0.5+0.2	6.0	1.0	1.0	1.0	0.5	1.0	2.6	2.31	0.25	+
0.5+0.5	13.0	1.5	1.5	2.0	0.7	1.3	2.6	2.33	0.27	+
0.5+1.0	13.0	1.0	1.0	1.0	0.8	1.4	2.3	2.25	0.26	+
0.5+2.0	6.0	1.0	1.0	1.0	.06	1.1	2.2	2.20	0.23	+
1.0+0.1	13.0	1.5	1.5	2.0	0.8	1.7	2.6	2.35	0.26	+
1.0+0.2	13.0	1.5	2.0	2.5	0.7	1.5	2.7	2.78	0.28	+
1.0+0.5	20.0	1.6	1.6	2.6	0.7	1.8	2.7	2.26	0.24	+
1.0+1.0	26.0	1.7	2.0	2.5	0.8	1.5	2.5	2.21	0.23	+
1.0+2.0	20.0	1.5	2.0	2.5	0.7	1.3	2.4	2.58	0.26	+
2.0+0.1	20.0	1.6	2.0	2.3	0.8	1.5	2.6	2.58	0.25	+
2.0+0.2	33.0	1.6	2.2	2.7	0.9	1.3	2.9	2.81	0.29	+
2.0+0.5	33.0	1.4	2.0	2.2	0.6	1.1	2.3	2.78	0.28	+
2.0+1.0	26.0	1.2	1.6	2.0	0.5	1.1	2.2	2.66	0.26	+

Contd.

Table 25 (Contd.)

2.0+2.0	20.0	1.0	1.5	1.5	0.5	1.0	2.0	2.20	0.23	+
3.0+0.1	26.0	1.2	1.7	2.0	0.6	1.3	2.1	1.98	0.20	+
3.0+0.2	26.0	1.0	1.7	2.0	0.6	1.2	2.0	1.99	0.21	+
3.0+0.5	33.0	1.4	1.6	2.2	0.7	1.2	2.0	1.98	0.21	+
3.0+1.0	20.0	1.5	1.5	2.0	0.5	1.0	1.9	1.95	0.20	+
3.0+2.0	6.0	1.0	1.0	1.0	0.4	0.9	1.6	1.87	0.19	+
$LSD_{5\%}$	8.5	NS	0.6	0.3	NS	0.5	0.4	0.22	0.13	
BA+ GA_3										
0.5+0.1	6.0	1.0	1.0	1.0	0.5	1.0	1.9	2.12	0.22	
0.5+0.2	6.0	1.0	1.0	1.0	0.5	1.2	2.0	2.16	0.23	
0.5+0.5	6.0	1.0	1.0	2.0	0.6	1.0	2.0	2.15	0.24	
0.5+1.0	13.0	1.0	1.5	2.0	0.6	1.0	2.1	2.23	0.23	
0.5+2.0	6.0	1.0	1.0	1.0	0.5	0.9	1.9	2.01	0.21	
1.0+0.1	13.0	1.5	1.5	2.0	0.8	1.7	2.9	3.01	0.31	-
1.0+0.2	13.0	1.5	1.5	2.0	0.7	1.5	2.7	2.90	0.30	-
1.0+0.5	20.0	1.5	1.6	2.0	0.8	1.4	2.6	2.81	0.29	-
1.0+1.0	20.0	1.2	1.5	2.0	0.7	1.5	2.5	2.65	0.27	-
1.0+2.0	13.0	1.0	1.0	1.0	0.5	1.1	2.1	2.35	0.25	-
2.0+0.1	26.0	1.2	1.5	1.7	0.6	1.2	2.0	2.45	0.26	-
2.0+0.2	40.0	1.5	2.0	2.3	0.5	1.2	2.1	2.40	0.25	-

Contd.

Table 25 (Contd.)

2.0+0.5	33.0	1.4	2.2	2.5	0.6	1.2	2.0	2.15	0.22	-
2.0+1.0	26.0	1.2	1.5	1.5	0.4	1.0	2.0	1.12	0.21	-
2.0+2.0	20.0	1.0	1.5	1.5	0.5	1.0	1.9	2.00	0.19	-
3.0+0.1	33.0	1.4	1.6	2.0	0.7	1.3	2.1	2.30	0.24	-
3.0+0.2	33.0	1.4	1.6	1.8	0.6	1.3	2.2	2.40	0.25	-
3.0+0.5	26.0	1.0	1.2	1.2	0.5	1.0	2.0	2.17	0.22	-
3.0+1.0	20.0	1.0	1.2	1.2	0.5	1.1	2.1	2.18	0.23	-
3.0+2.0	6.0	1.0	1.0	1.0	0.4	0.8	1.9	2.15	0.22	-
4.0+0.1	26.0	1.2	1.2	1.5	0.6	0.9	1.7	1.98	0.20	-
4.0+0.2	20.0	1.0	1.2	1.2	0.4	1.0	1.8	1.99	0.21	-
4.0+0.5	20.0	1.2	1.2	1.5	0.5	1.0	1.7	1.95	0.20	-
4.0+1.0	13.0	1.0	1.5	1.5	0.3	0.9	1.5	1.85	0.19	-
4.0+2.0	6.0	1.0	1.0	1.0	0.3	0.7	1.3	1.81	0.18	-
$LSD_{5\%}$	18.5	NS	NS	0.3	NS	NS	0.4	0.26	0.13	

The lowest average number of shoots per culture was obtained when 0.5 mg/l BA was supplemented with 0.1, 0.2 and 2.0 mg/l NAA and 3.0 mg/l BA supplemented with 2.0 mg/l NAA. Elongation of shoots was noted in these cultures only. In the other BA and NAA supplemented media, average number of shoots per culture was always less than 2 at 15 days of culture. Shoot number increased to 2 after 45 days of culture (Plate–II, B). The number of shoots per culture varied greatly

on different media composition in respect of BA and NAA. Higher number of shoots per explant was recorded in 0.5, 1.0 and 2.0 mg/l BA supplemented media. The highest number of shoots per explant was 2.9 in 2.0 mg/l BA + 0.2 mg/l NAA supplemented media.

Average length of the longest shoot was always less than 1 cm at 15 days of culture that gradually increased to almost 3 cm in some media composition of 45 days of culture. The longest shoot was recorded in media composition where 2.0 mg/l BA + 0.2 mg/l NAA were added and it was 2.9 cm on average. 1.0 mg/l BA with 5 concentrations of NAA gave uniform shoot length which ranged from 2.4 cm to 2.7 cm.

Fresh weight of shoot was lowest in 3.0 mg/l BA with all the concentrations of NAA and it ranged from 1.87 to 1.99 g per culture. The fresh weight per culture was significantly greater in 2.0 mg/l BA compared to 0.1 mg/l and 0.5 mg/l with all the 5 concentrations of NAA used. The highest fresh weight was 2.81 g per culture in media having 2.0 mg/l BA + 0.2 mg/l NAA.

Dry weight of shoots per culture was also high in 2.0 mg/l BA that was significantly greater than those cultured in other concentrations of BA supplemented media. The highest dry weight was 0.29 g in media where 2.0 mg/l BA + 0.2 mg/l NAA were used. The dry weight of shoots was 0.28 g in 1.0 mg/l BA + 0.2 mg/l NAA and 2.0 mg/l BA + 0.5 mg/l NAA supplemented media.

Base callusing was recorded in all the culture media used.

Effect of different concentrations of BA and GA_3.

Effect of BA with different concentrations of GA_3 on multiple shoot induction from shoot tip explants was studied in this experiment. Five different concentrations

of BA (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) were used with 5 GA_3 concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l).

Data collected or percentage of cultures induced multiple shooting, number of shoots per explant, mean length of longest shoots in a culture are given in Table 25.

Percentage of cultures responded to shoot elongation and multiple shooting depended on the BA and GA_3 concentrations in the culture media. The lowest percentage of cultures responded in 0.5 mg/l BA and it ranged from 6% in 0.5 mg/l BA + 0.1 mg/l GA_3 to 13% in 0.5 mg/l BA + 1.0 mg/l GA_3. The highest percentage of cultures responded to shoot elongation and multiple shooting was recorded in media with 2.0 mg/l BA + 0.2 mg/l GA_3 where it was 40%.

Mean number of shoots per explant was 1 at 15 days of culture in most of the media where 0.5 mg/l BA with 0.1, 0.2, and 2.0 mg/l GA_3 were used. Number of shoots per explant was also 1 in media where 1.0 mg/l BA + 2.0 mg/l GA_3, 3.0 mg/l BA + 2.0 mg/l GA_3 and 4.0 mg/l BA + 2.0 mg/l GA_3 were used. The highest number of shoots per explant was noted in media where 2.0 mg/l BA + 0.5 mg/l GA_3 was used and which was 2 shoots at 45 days of culture (Plate – II, D). On average, the number of shoots per culture was highest in 2.0 mg/l BA used in the media. Significant effect of GA_3 was also noted in respect of shoot number per culture with all the 5 levels of BA used in this experimentation.

The average length of the longest shoot was found to be influenced by the concentrations of BA used. GA_3 was also found to play some role in shoot elongation. The highest shoot length was recorded in media where 1 mg/l BA + 0.1 mg/l GA_3 were used which was 2.9 cm. The highest concentrations of BA with all the 5 concentrations of GA yielded the lowest average length of shoots per culture

Fresh weight of shoots per explant was greater in the culture media containing 1.0 mg/l BA compared to other BA concentrations used. The fresh weight of shoot was lowest in 4.0 mg/l BA containing media.

Dry weight of shoots was significant in 1.0 mg/l BA with all the 5 concentrations of GA_3 used in the culture media. GA_3 also showed significant effect when used with BA on dry weight of shoots per explant. Dry weight of shoots was always low when cultured in media having 4.0 mg/l BA with all the 5 concentrations of GA_3.

Effect of Different Concentrations and Combinations of Kn With NAA and GA_3 on Shoot Multiplication from *in vitro* Grown Shoot Tip

Effect of different concentrations of Kn and NAA

Effect of different concentrations of Kn with various concentrations of NAA on shoot multiplication from shoot tip explant was studied in this experiment. Brief description of this experiment is given below:

Six different concentrations of Kn (0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) were used with 4 concentrations (0.1, 0.5, 1.0 and 2.0 mg/l) of NAA.

Data collected on percentage of cultures responded to multiple shoots, mean shoot number per explant, mean length of longest shoot per culture and fresh and dry weights of shoots per culture are shown in Table 26.

Table 26 : Effect of different concentrations and combinations of Kn with NAA and GA_3 on shoot multiplication from *in vitro* grown shoot tip of *R. serpentina.*

Growth regul-ators (mg/l)	% of shoot forma -tion	Average no. of shoots/explant after days			Average shoot length (cm). after days			Average wt of shoots/ explant (g) after 45 days		Base Callu sing
		15	30	45	15	30	45	Fresh wt.	Dry wt.	
Kn+NAA										
0.5+0.1	7.0	1.0	1.0	2.0	0.6	1.3	2.4	2.54	0.26	-
0.5+0.5	7.0	1.0	1.0	2.0	0.5	1.1	1.4	2.53	0.25	-
0.5+1.0	7.0	1.0	2.0	2.0	0.5	1.2	2.5	2.68	0.27	-
0.5+2.0	14.2	1.5	2.0	2.2	0.4	1.0	2.3	2.51	0.26	-
1.0+0.1	14.2	2.0	2.5	2.5	0.7	1.3	2.5	2.60	0.27	-
1.0+0.5	21.4	2.3	2.6	2.6	0.7	1.2	1.5	2.60	0.26	-
1.0+1.0	28.5	2.0	2.3	2.5	0.6	1.0	2.2	2.50	0.24	-
1.0+2.0	21.4	2.0	2.3	2.7	0.5	1.1	2.3	2.41	0.24	-
2.0+0.1	28.4	2.3	2.5	2.7	0.8	1.5	2.9	2.81	0.29	-
2.0+0.5	35.7	2.4	2.6	2.6	0.7	1.5	2.8	2.78	0.29	-
2.0+1.0	35.7	2.2	2.5	2.6	0.6	1.2	2.5	2.48	0.25	-
2.0+2.0	43.0	2.3	2.6	2.8	0.7	1.3	2.7	2.71	0.27	-
3.0+0.1	50.0	2.4	2.6	2.9	0.8	1.8	3.1	3.10	0.33	-

Contd.

Table 26 (Contd.)

3.0+0.5	57.1	2.3	2.4	2.9	0.6	1.7	3.0	3.02	0.31	-
3.0+1.0	64.0	2.4	2.9	3.1	0.5	0.6	3.0	2.98	0.30	-
3.0+2.0	35.7	2.2	2.4	2.8	0.7	1.4	2.9	2.87	0.29	-
4.0+0.1	35.7	2.4	2.6	2.6	0.6	1.4	2.7	2.71	0.27	-
4.0+0.5	35.7	2.4	2.6	2.6	0.6	1.3	2.7	2.75	0.28	-
4.0+1.0	21.4	2.0	2.3	2.3	0.5	1.0	2.3	2.38	0.28	-
4.0+2.0	7.0	1.0	2.0	2.0	0.6	1.1	2.4	2.45	0.24	-
$LSD_{5\%}$	16.2	NS	NS	0.5	NS	NS	0.5	0.18	0.10	
Kn + GA_3										
0.5+0.1	6.0	1.0	1.0	2.0	0.4	0.9	2.0	2.61	0.27	-
0.5+0.5	6.0	1.0	1.0	2.0	0.3	0.8	2.0	2.65	0.27	-
0.5+1.0	12.5	1.5	2.0	2.0	0.5	1.2	2.3	2.74	0.29	-
0.5+2.0	19.0	2.0	2.3	2.3	0.5	1.2	2.2	2.71	0.28	-
1.0+0.1	12.5	1.5	1.5	2.0	0.6	1.3	2.4	2.69	0.27	-
1.0+0.5	12.5	1.5	2.0	2.0	0.5	1.2	2.3	2.72	0.28	-
1.0+1.0	25.0	1.8	1.8	2.2	0.4	1.0	2.2	2.72	0.27	-
1.0+2.0	31.2	1.8	2.0	2.2	0.3	0.9	1.7	2.46	0.25	-
2.0+0.1	25.0	1.5	2.0	2.3	0.4	1.0	2.0	2.65	0.28	-
2.0+0.5	31.2	1.6	1.8	2.0	0.5	1.2	2.1	2.71	0.28	-

Contd.

Table 26 (Contd.)

2.0+1.0	37.5	1.8	2.0	2.1	0.6	1.5	2.5	2.91	0.29	-
2.0+2.0	25.0	1.8	1.8	2.0	0.4	1.0	1.9	2.66	0.27	-
3.0+0.1	31.2	1.4	1.6	2.0	0.5	1.1	1.9	1.69	0.18	-
3.0+0.5	31.2	1.2	1.4	1.8	0.5	1.2	2.1	2.64	0.28	-
3.0+1.0	25.0	1.5	1.5	1.8	0.6	1.3	2.3	2.75	0.28	-
3.0+2.0	12.5	1.0	1.5	1.5	0.3	0.8	2.0	2.53	0.26	-
4.0+0.1	25.0	1.2	1.5	1.8	0.4	0.9	2.1	2.51	0.25	-
4.0+0.5	12.5	1.0	1.5	1.5	0.4	1.0	2.0	2.57	0.26	-
4.0+1.0	12.5	1.0	1.5	1.5	0.3	0.8	1.8	1.59	0.16	-
4.0+2.0	6.0	1.0	1.5	1.5	0.3	0.7	1.7	1.56	0.15	-
$LSD_{5\%}$	10.2	NS	0.4	0.6	NS	0.4	0.3	0.12	0.08	

The highest percentage of explants responded to multiple shooting was achieved when cultured on media having 3.0 mg/l Kn with 0.1–1.0 mg/l NAA. The lowest percentage of the cultures responded to multiple shooting in media where 0.5 mg/l Kn with all the 4 concentrations of NAA was used. The cultures did not respond to induce multiple shooting in 0.1 mg/l Kn.

The range of cultures responded to multiple shooting was 7.0–14.2%, 14.2–28.5%, 28.4–43%, 35.7–64% and 7.0–35.7% respectively in 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l Kn with all the 4 concentrations of NAA. As high as 64% of cultures responded

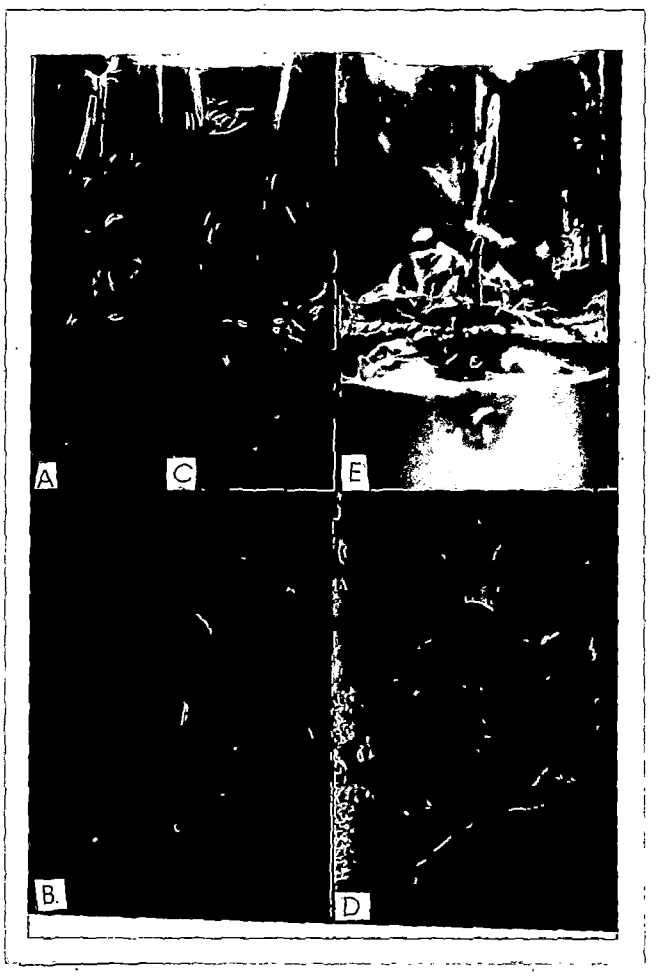


Plate II

A. Shoot formation in medium having MS + 2.0 mg/l BA from *in vitro* grown shoot tip.

B. Shoot formation in medium having MS + 2.0 mg/l BA + 0.2 mg/ l NAA.

C. Shoot formation in medium having MS + 3.0 mg/l Kn + 1.0 mg/ l NAA.

D. Shoot formation in medium having MS + 2.0 mg/l BA + 0.5 mg/ l GA_3.

E. Shoot formation in medium having MS + 2.0 mg/l Kn + 0.1 mg/l GA_3.

to multiple shooting when 3.0 mg/l BA + 1.0 mg/l NAA was used in the culture media.

Average number of shoots per explant was always greater than 2 or less than 3 in any of the culture media used for multiple shooting. There was a gradual increase in shoot number from 15 days to 45 days for all the culture media used. The highest number of shoots per explant was noted in media where different concentrations of NAA was used with 3.0 mg/l Kn and it ranged from 2.8–3.1 shoots/culture (Plate–II, C). The lowest number of shoots was obtained when 0.5 mg/l Kn was used and it ranged from 2.0–2.2 per culture at 45 days of culture. Other Kn concentrations with different NAA concentrations had moderate number of shoots per explant ranging from 2 to 2.8 shoots.

Average length of shoots was also influenced by the concentrations of Kn used in the media. Significant differences among the different concentrations of NAA used within a Kn a concentration was noted. Shoot length was found to increase gradually from 15 days of culture to 45 days of culture and it ranged from 1.4–3.1 cm. The length of shoots was highest on media where 3.0 mg/l Kn was used with various concentrations of NAA and it ranged from 2–9 cm in 3.0 mg/l Kn + 2.0 mg/l NAA to 3.1 cm in 3.0 mg/l Kn + 0.1 mg/l NAA.

Fresh weight of shoots per culture was highly affected by concentrations of Kn used in the media. On average, the fresh weight was greater in 3.0 mg/l Kn compared to other concentrations. The highest fresh weight of shoots was 3.10 g/culture in media where 3.0 mg/l Kn was used.

Dry weight of shoots was influenced by the concentrations of Kn as well as NAA concentrations within a Kn used in the media. Dry weight of shoots per explant

was significantly greater in 3.0 mg/l Kn compared to other concentrations of Kn used. The highest dry weight of shoots was 0.33 g per culture in 3.0 mg/l Kn + 1.0 mg/l NAA.

Effect of different concentrations of Kn and GA_3

Shoot tips were cultured for multiple shooting in Kn and GA_3 supplemented media. Six different concentrations of Kn (0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) were used with 4 different concentrations of NAA (0.1, 0.5, 1.0 and 2.0 mg/l).

Results obtained for percentage of culture responded to multiple shooting, mean number of shoots per culture, mean length of the longest shoots and fresh and dry weight of shoots per culture are presented in Table 26.

The highest response of multiple shooting ranged from 37.5% in 2.0 mg/l Kn + 1.0 mg/l GA_3 whereas the lowest percentage of culture response was 6% in media supplemented with 0.5 mg/l Kn + 0.1 mg/l GA_3, 0.5 mg/l Kn + 0.5 mg/l GA_3 and 4.0 mg/l Kn + 2.0 mg/l GA_3.

Average number of shoots per culture was always less than 2 per culture at 15 days of culture in each of the combinations of Kn and GA_3. It gradually increased and the highest number of shoots per culture was recorded at 45 days of culture which was 2.3 in media supplemented with 0.5 mg/l Kn + 2.0 mg/l GA_3 and 2.0 mg/l Kn + 0.1 mg/l GA_3 (Plate–II, E). Higher concentrations of GA_3 failed to induce multiple shoots at 45 days of culture.

Average length of the longest shoot was less than 1 cm in all the combinations of Kn and GA_3 at 15 days of culture but in most of the culture media it was about 2 or more than 2 at 45 days of culture. The length of the longest shoot was 2.5 cm in 2.0

mg/l Kn + 1.0 mg/l GA_3 found to be 1.7 cm in 1.0 mg/l Kn + 2.0 mg/l GA_3 and 4.0 mg/l Kn + 2.0 mg/l GA_3.

Fresh weight of shoots was also influenced by the hormonal concentrations used. Fresh weight of shoots gradually decreased with the increase of Kn concentrations. The highest fresh weight was obtained in media supplemented with 2.0 mg/l Kn + 1.0 mg/l GA_3 and the lowest fresh weight was obtained in 4.0 mg/l Kn + 2.0 mg/l GA_3.

The highest dry weight obtained was 0.29 g/culture in media where 2.0 mg/l Kn + 1.0 mg/l GA_3 was used. The lowed dry weight was 0.15 g per culture in 4.0 mg/l Kn + 2.0 mg/l GA_3.

CALLUS INDUCTION IN DIFFERENT COMBINATIONS AND CONCENTRATIONS OF PHYTOHORMONES

This part of the study includes several experiments on induction of callus from internode and leaves of *in vitro* grown shoots.

Effect of Different Concentrations of Auxins on Callus Induction from *in vitro* Grown Nodal Segment

In this experiment different types of auxins viz., IAA, IBA, NAA and 2,4-D were included to induce calluses from internode of *in vitro* grown shoots. The concentrations of auxins were 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l.

Explants started to induce callus within 7 days of culture and continued to increase its size up to 60 days. All the four auxins induced calluses when culture media contained 1 to 5.0 mg/l of the respective auxin. Less than 1.0 mg/l auxin failed to induce any callus.

Data obtained on percentage of cultures induced callus, type of callus and fresh and dry weights of callus were recorded and are presented in Table 27. Highly significant differences were noted between the types of auxins as well as between auxin concentrations in culture media within a type of auxin. Among the four types of auxins, 2,4-D had the highest callus inducing effect compared to other three auxins studied (Plate–III, A). Auxins NAA, IBA and IAA had more or less equal effect on callus induction. Percentage of cultures induced callus depended on the concentration of auxin used. For 2,4-D the lowest of 41.66% of cultures induced callus in 5.0 mg/l, whereas the highest of 83.33% of cultures induced callus in 3.0 mg/l of 2,4-D.

The range of percentage of cultures induced callus in NAA supplemented media was 16.66% in 5.0 mg/l and 41.66% in 3.0 mg/l NAA. For IBA, the lowest of 25% of cultures induced callus in both 1.0 mg/l and 5.0 mg/l IBA, whereas the highest percentage of cultures induced callus in 4.0 mg/l IBA that was 41.66%.

For IAA the range of culture induced calluses was 16.66% in 1.0 mg/l to 41.66% in 4.0 mg/l IAA.

The nature of callus induced was hard in texture and white in colour in all the four types of auxins used in the media. Regeneration of shoots from callus cultures was not observed in any of the culture media used.

Fresh weight of callus was taken after 60 days of culture and is shown in Table 27. The fresh weight of callus was highest in 2,4-D containing media.

Significant differences between callus weight induced in different concentrations and types of auxins used were observed. The callus weight obtained from NAA, IBA and IAA were more or less similar and no significant differences among these three types of auxins was observed. The range of callus weight was 2.80 g per culture in 5.0 mg/l to 3.64 g per culture in 3.0 mg/l 2,4-D. Its range in NAA containing medium was 1.58 g/culture in 5.0 mg/l to 2.55 g/culture in 3.0 mg/l. The lowest callus weight was obtained in 5.0 mg/l IBA and it was 1.59 g/culture, whereas the highest callus weight was 2.35 g per culture as obtained in 4.0 mg/l IBA.

Table 27 : Effect of different concentration of auxins on callus induction from *in vitro* grown nodal segment of *R. serpentina.*

Growth regulators(mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
IAA				
1.0	16.66	1.78	0.09	White
2.0	25.00	2.00	0.10	White
3.0	33.33	1.98	0.10	White
4.0	41.66	2.40	0.12	White
5.0	25.00	2.20	0.11	White
$LSD_{5\%}$	12.6	0.18	NS	
IBA				
1.0	25.00	1.99	0.10	White
2.0	33.33	2.19	0.11	White

Contd.

Table 27 (Contd.)

3.0	33.33	2.17	0.11	White
4.0	41.66	2.35	0.12	White
5.0	25.00	1.59	0.08	White
$LSD_{5\%}$	10.5	0.5	NS	
NAA				
1.0	33.33	2.18	0.11	White
2.0	33.33	2.19	0.11	White
3.0	41.66	2.55	0.13	White
4.0	25.00	1.97	0.10	White
5.0	16.66	1.58	0.08	White
$LSD_{5\%}$	13.5	NS	NS	
2, 4 - D				
1.0	58.33	2.95	0.14	White
2.0	66.66	3.00	0.15	White
3.0	83.33	3.64	0.19	White
4.0	75.00	3.51	0.18	White
5.0	41.66	2.80	0.14	White
$LSD_{5\%}$	16.5	0.50	NS	

Calli obtained were dried at 55°C for 5 days in a temperature-controlled dryer and the dry weights obtained are presented in Table 27.

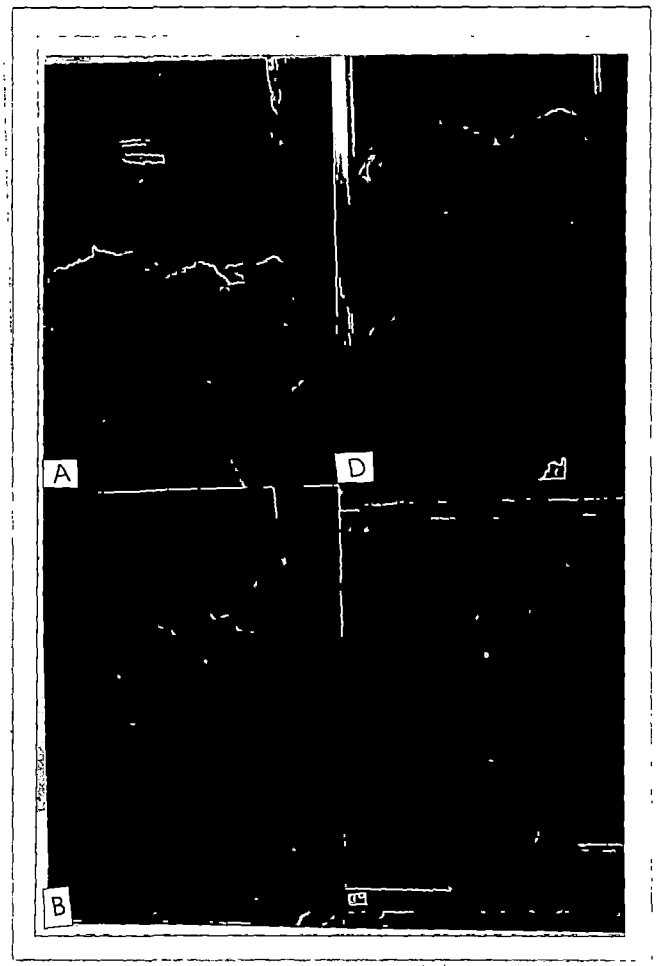


Plate III

A. Hardy-white compact callus obtained from medium having MS + 3.0 mg/l 2.4-D.

B. Hardy-white compact callus obtained from medium having MS + 2.0 mg/l 2.4-D + 0.5 mg/l BA.

C. Hardy-white compact callus obtained from medium having MS + 2.0 mg/l 2.4-D + 1.0 mg/l Kn.

D. Hardy-white compact callus obtained from medium having MS + 2.0 mg/l 2.4-D + 0.5 mg/l BA + 0.1 mg/l CH.

Higher dry weight per callus was noted in 2,4-D supplemented medium. The highest dry weight of 0.19 g and 0.18 g were obtained in 3.0 and 4.0 mg/l 2,4-D, respectively. The highest dry weight of calli in NAA, IBA and IAA were 0.13, 0.12 and 0.12 g, respectively. Dry weight between different auxins and within a type of auxin showed significant differences among themselves.

Effect of Different Concentrations and Combinations of 2,4-D with BA on Callus Induction from *in vitro* Grown Nodal Segment

In this experiment 2, 4-D in combination with BA was used to induce callus and callus growth was studied after 60 days of culture. Different concentrations of 2, 4-D were 0.5, 1.0, 2.0 and 3.0 mg/l and different concentrations of BA were 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l.

Results obtained on callus induction, nature of callus, fresh and dry weights of callus are shown in Table 28.

It was observed that 2.0 mg/l 2.4-D had the highest effect on callus induction compared to other concentrations of 2, 4-D. Hundred percent of the cultures induced callus when cultured on medium with 2.0 mg/l 2, 4-D + 0.5 mg/l BA (Plate – III, B). The lowest percentage of cultures induced callus on media with 0.5 mg/l 2, 4-D with all the concentrations of BA.

The nature of callus obtained was white in colour and hard in texture. Average fresh callus weight was highest when cultured on media having 2.0 mg/l 2, 4-D with all the 5 concentrations of BA. The highest fresh weight of callus was obtained from culture having 2.0 mg/l 2, 4-D + 0.5 mg/l BA. The results indicated that 2.0 mg/l yielded the highest amount of dry callus weight.

Table 28 : Effect of different concentrations and combinations of 2, 4 – D with BA on callus induction from *in vitro* grown nodal segment of *R. serpentina*.

Growth regulators (mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2,4–D + BA				
0.5+0.1	64.28	3.62	0.18	White
0.5+0.5	64.28	3.64	0.18	White
0.5+1.0	71.42	3.81	0.19	White
0.5+2.0	64.28	3.63	0.18	White
0.5+3.0	57.14	3.60	0.18	White
1.0+0.1	71.42	3.79	0.19	White
1.0+0.5	71.42	3.80	0.20	White
1.0+1.0	78.57	3.97	0.19	White
1.0+2.0	71.42	3.78	0.19	White
1.0+3.0	64.28	3,58	0.18	White
2.0+0.1	85.71	3.99	0.20	White
2.0+0.5	100.00	4.15	0.21	White
2.0+1.0	92.85	4,13	0.21	White
2.0+2.0	85.71	3.96	0.20	White
2.0+3.0	78.57	3.93	0.20	White
3.0+0.1	85.71	3.97	0.20	White

Contd.

Table 28 (Contd.)

3.0+0.5	85.71	3.96	0.20	White
3.0+1.0	78.57	3.79	0.19	White
3.0+2.0	64.28	3.58	0.18	White
3.0+3.0	57.14	3.42	0.17	White
$LSD_{5\%}$	26.5	0.7	NS	

Effect of Different Concentrations and Combinations of 2, 4-D with Kn on Callus Induction from *in vitro* Grown Nodal Segment

In this experiment 2, 4-D supplemented with Kn was used to induce callus and callus growth was studied. Different concentrations of 2, 4- D (0.5, 1.0, 2.0 and 3.0 mg/l) and 5 different concentrations (0.1, 0.5, 1.0, 2.0, and 3.0 mg/l) of Kn were used.

Results obtained on callus induction, nature of callus, fresh and dry weights of callus are presented in Table 29.

2.0 mg/l 2,4-D had the highest effect on callus induction compared to other concentrations of 2, 4-D. Hundred percent of cultures induced callus when cultured on medium having 2.0 mg/l 2, 4-D + 1.0 mg/l Kn (Plate–III, C). The lowest percentage of cultures induced callus on media containing 3.0 mg/l 2, 4-D irrespective of concentrations of Kn used. It was observed that 2, 4-D with Kn enhanced callus induction. The nature of callus as obtained was white in colour and hard in texture.

Table 29 : Effect of different concentrations and combinations of 2, 4 – D with Kn on callus induction from *in vitro* grown nodal segment of *R. serpentina.*

Growth regulators (mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2, 4 – D + Kn				
0.5+0.1	66.67	3.56	0.18	White
0.5+0.5	57.00	3.68	0.18	White
0.5+1.0	71.00	3.69	0.18	White
0.5+2.0	75.00	3.51	0.17	White
0.5+3.0	67.00	3.52	0.17	White
1.0+0.1	71.00	3.74	0.18	White
1.0+0.5	75.00	3.76	0.18	White
1.0+1.0	77.00	3.78	0.18	White
1.0+2.0	70.00	3.69	0.17	White
1.0+3.0	67.00	3.65	0.17	White
2.0+0.1	83.33	3.88	0.19	White
2.0+0.5	91.00	3.91	0.19	White
2.0+1.0	100.00	4.16	0.21	White
2.0+2.0	95.00	4.00	0.20	White
2.0+3.0	83.33	3.91	0.19	White
3.0+0.1	58.00	3.53	0.17	White
3.0+0.5	66.66	3.61	0.17	White

Contd.

Table 29 (Contd.)

3.0+1.0	66.66	3.65	0.17	White
3.0+2.0	66.66	3.63	0.17	White
3.0+3.0	50.00	3.44	0.16	White
$LSD_{5\%}$	22.5	0.8	NS	

2,4-D had significant effect on the fresh weight of callus. Average fresh callus weight was high in medium containing 2.0 mg/l 2, 4-D with all the 5 concentrations of Kn. The highest fresh weight of callus was obtained from culture containing 2.0 mg/l 2,4–D + 1.0 mg/l Kn.

The dry weight of callus was maximum in 2.0 mg/l 2,4-D and it ranged from 0.19 g to 0.21 g. The lowest dry weight was obtained when 3.0 mg/l 2, 4-D was used with all 5 concentrations of Kn.

Effect of Different Concentrations and Combinations of 2, 4-D with BA and CH on Callus Induction from *in vitro* Grown Nodal Segment

In this experiment 2, 4-D in combination with BA as well CH was used to induce callus and callus growth was studied. 2.0 mg/l 2, 4-D was used with 4 different concentrations of BA (0.1, 0.5, 1.0 and 2.0 mg/l) and 5 different concentrations of CH (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l).

Results obtained on callus induction, nature of callus, fresh and dry weights of callus are presented in Table 30.

Table 30 : Effect of different concentrations and combinations of 2, 4 – D with BA and CH on callus induction from *in vitro* grown nodal segment of *R. serpentina.*

Growth regulators(mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2,4–D + BA + CH				
2.0+0.1+0.1	88.23	4.25	0.22	White
2.0+0.1+0.5	88.23	4.38	0.23	White
2.0+0.1+1.0	94.11	4.39	0.23	White
2.0+0.1+2.0	88.23	4.28	0.22	White
2.0+0.1+3.0	82.35	4.12	0.21	White
2.0+0.5+0.1	100.00	4.54	0.24	White
2.0+0.5+0.5	100.00	4.53	0.24	White
2.0+0.5+1.0	94.11	4.37	0.23	White
2.0+0.5+2.0	88.23	4.29	0.22	White
2.0+0.5+3.0	82.35	3.91	0.20	White
2.0+1.0+0.1	94.11	4.31	0.22	White
2.0+1.0+0.5	94.11	4.33	0.22	White
2.0+1.0+1.0	88.23	4.14	0.21	White
2.0+1.0+2.0	82.35	4.13	0.21	White
2.0+1.0+3.0	76.47	3.75	0.19	White
2.0+2.0+0.1	88.23	3.95	0.20	White

Contd.

Table 30 (Contd.)

2.0+2.0+0.5	82.35	3.96	0.20	White
2.0+2.0+1.0	76.35	3.81	0.19	White
2.0+2.0+2.0	76.35	3.93	0.20	White
2.0+2.0+3.0	70.58	3.75	0.19	White
$LSD_{5\%}$	26.3	1.10	NS	

Irrespective of concentrations of BA and CH, 2.0 mg/l 2, 4-D showed maximum effect on callus induction in this experiment. Above all 0.5 mg/l BA and 0.1 to 0.5 g/l CH with 2, 4-D had the highest effect on callus induction compared to other concentration of BA and CH. Hundred percent of the cultures induced callus when cultured on media having 2.0 mg/l 2, 4-D + 0.5 mg/l BA +0.1 g/l CH and 2.0 mg/l 2,4 – D + 0.5 mg/l BA + 0.5 g/l CH (Plate – III, D). The lowest percentage of cultures induced callus on medium when 2.0 mg/l 2, 4-D + 0.5 mg/l BA + 3.0 mg/l CH were used. The nature of callus obtained was white in colour and hard in texture.

Fresh weight of callus also showed marked effect on BA concentrations with CH used in culture media. Average callus weight was high when cultured on media having 2.0 mg/l 2, 4-D and 0.5 mg/l BA with all the 5 concentrations of CH. The highest fresh weight of callus was obtained from culture having 2.0 mg/l 2, 4 +D + 0.5 mg/l BA + 0.1 mg/l.

The highest dry weight was obtained when 0.1 mg/l and 0.5 mg/l CH was used with 2.0 mg/l 2, 4-D + 0.5 mg/l BA.

Effect of Different Concentrations of Auxins on Callus Induction from *in vitro* Grown Leaf Segment

In this experiment different types of auxins i.e. IBA, NAA and 2, 4-D were included to induce callus from leaves of *in vitro* grown plantlets. The concentrations of each of the four auxins were 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l.

The explants started to induce callus within 7 days of culture and continued to increase its size up to 60 days. All the four auxins induced callus when culture media contained 1.0 to 5.0 mg/l of the respective auxins. Less than 1.0 mg/l auxin failed to induce any callus.

Data obtained on percentage of cultures induced callus is shown in Table 31. Highly significant differences were noted between auxin concentrations in culture media within a type of auxin. Among the four types of auxins, 2, 4-D had highest callus inducing effect compared to other three auxins studied (Plate–IV, A). Auxins NAA, IBA and IAA had different effect on callus induction as indicated in Table 31. The lowest percentage of culture induced to callus in IBA containing media. Percentage of cultures induced callus depended on the concentrations of auxin used. For 2, 4-D, the lowest percentage of callus induction was recorded as 33.33% in 5.0 mg/l, whereas the highest was recorded as 66.66% in 3.0 mg/l.

In NAA supplemented media the lowest callus induction was 16.66% in 1.0 mg/l and the highest was 41.66% in 3.0 mg/l. For IAA the lowest of 16.66% of culture induced callus in 1.0 mg/l and the highest of 33.33% in 4.0 mg/l. The range of callus induction was 8.33% in 1.0 mg/l to 25% in 4.0 mg/l in IBA.

Fresh and dry weights of callus were taken after 60 days of culture and shown in Table 31. The fresh weight of callus was highest in 2, 4-D supplemented media.

Plate IV

A. Greenish callus obtained from medium having MS + 3.0 mg/l 2.4-D.

B. Greenish callus obtained from medium having MS + 2.0 mg/l 2.4-D + 1.0 mg/l BA.

C. Greenish callus obtained from medium having MS + 2.0 mg/l 2.4-D + 1.0 mg/l Kn.

D. Greenish callus obtained from medium having MS + 2.0 mg/l 2.4-D + 0.5 mg/l Kn + 0.1 g/l CH..

Table 31 : Effect of different concentrations of auxins on callus induction from *in vitro* grown leaf segments of *R. serpentina*.

Growth regulators (mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2, 4 - D				
1.0	41.66	2.38	0.11	Greenish
2.0	50.00	2.66	0.12	Greenish
3.0	66.66	2.69	0.12	Greenish
4.0	58.33	2.60	0.12	Greenish
5.0	33.33	2.41	0.11	Greenish
$LSD_{5\%}$	14.5	NS	NS	
NAA				
1.0	16.66	1.11	0.05	Greenish
2.0	33.33	2.49	0.11	Greenish
3.0	41.66	2.61	0.11	Greenish
4.0	25.00	2.15	0.09	Greenish
5.0	25.00	1.80	0.08	Greenish
$LSD_{5\%}$	12.6	0.8	NS	
IAA				
1.0	16.66	1.15	0.05	Greenish
2.0	16.66	2.49	0.11	Greenish

Contd.

3.0	25.00	2.60	0.12	Greenish
4.0	33.33	2.41	0.11	Greenish
5.0	16.66	1.19	0.05	Greenish
$LSD_{5\%}$	12.5	0.5	0.02	
IBA				
1.0	8.33	1.02	0.04	Greenish
2.0	16.66	2.41	0.11	Greenish
3.0	16.66	2.56	0.11	Greenish
4.0	25.00	2.60	0.12	Greenish
5.0	16.66	1.12	0.05	Greenish
$LSD_{5\%}$	8.5	0.50	0.03	

There were differences between types of auxins for fresh weight. The range of fresh callus weight was 2.38 g/culture in 1.0 mg/l to 2.69 g/culture in 3.0 mg/l 2, 4-D. In NAA, IAA and IBA containing media it was 1.11 g in 1.0 mg/l to 2.61 g/culture in 4.0 mg/l, 1.15 g in 1.0 mg/l to 2.60 g/culture in 4.0 mg/l and 1.02 g to 2.60 g/culture in 4.0 mg/l, respectively.

Higher dry weight per culture was noted in 2, 4-D supplemented medium and this weight in NAA, IBA and IAA supplemented media varied less. But dry weight between different concentrations used within a type of auxin showed marked

differences among themselves. The highest dry weight of 0.12 g/culture was obtained in media containing 1.0, 2.0 and 4.0 mg/l of 2, 4-D. The highest dry weight of callus in NAA, IBA and IAA was 0.11 g.

Effect of Different Concentrations of 2,4-D with BA on Callus Induction from *in vitro* Grown Leaf Segment

In this experiment 2, 4-D was supplemented with BA to induce callus and callus growth was studied. Four different concentrations of 2, 4-D (0.5, 1.0, 2.0 and 3.0 mg/l) and 5 concentrations of BA (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l) were used.

Results obtained on callus induction, nature of callus, fresh and dry weights of callus are shown in Table 32.

Marked effect of 2,4-D with BA was observed in all the culture media in this experiment. Above all, 2.0-mg/l 2,4-D, had the highest effect on callus induction compared to other concentrations of 2, 4-D used. The highest percentage (71.42) of cultures induced callus was observed in medium having 2.0 mg/l 2, 4-D + 1.0 mg/l BA (Plate – IV, B). On the other hand, the lowest percentage of callus was induced on media containing 0.5 mg/l 2, 4-D with all the concentrations of BA used. It was noted that 2, 4-D with BA enhanced callus induction. Marked differences among the concentrations of 2, 4-D as well as between different concentrations of BA within a 2, 4-D concentration were found.

2, 4-D concentrations used in the culture media showed a significant effect on fresh weight of callus. The highest fresh weight (3.76 g) of callus was obtained from culture medium having 2.0 mg/l 2, 4- D + 1.0 mg/l BA. On average, 2.0 mg/l 2, 4-D had greater callus weight compared to other concentrations of 2, 4-D used.

Table 32. Effect of different concentrations of 2, 4 – D with BA on callus induction from *in vitro* grown leaf segments of *R. serpentina.*

Growth regulators(mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2,4–D + BA				
0.5+0.1	35.71	3.18	0.15	Greenish
0.5+0.5	35.71	3.16	0.15	Greenish
0.5+1.0	42.85	3.38	0.16	Greenish
0.5+2.0	57.14	3.55	0.17	Greenish
0.5+3.0	50.00	3.58	0.17	Greenish
1.0+0.1	42.85	3.33	0.16	Greenish
1.0+0.5	42.85	3.35	0.16	Greenish
1.0+1.0	57.14	3.58	0.17	Greenish
1.0+2.0	57.14	3.56	0.17	White
1.0+3.0	50.02	3.47	0.17	White
2.0+0.1	50.00	3.53	0.16	White
2.0+0.5	57.15	3.70	0.18	White
2.0+1.0	71.42	3.76	0.18	White
2.0+2.0	64.28	3.65	0.17	White
2.0+3.0	57.14	3.59	0.17	White
3.0+0.1	57.14	3.56	0.15	White
3.0+0.5	64.28	3.59	0.16	White
3.0+1.0	57.14	3.61	0.16	White
3.0+2.0	42.85	3.62	0.16	White
3.0+3.0	35.71	3.55	0.15	White
$LSD_{5\%}$	20.5	NS	NS	

The dry weight of callus was also higher in 2.0 mg/l 2, 4-D + 1.0 mg/l BA. The lowest dry weight was found in the media having 0.5 mg/l 2, 4-D + 0.1 BA, 0.5 mg/l 2, 4-D + 3.0 mg/l BA.

The nature of callus obtained was greenish and hard in texture.

Effect of Different Concentrations of 2, 4-D with Kn on Callus Induction from *in vitro* Grown Leaf Segment

In this experiment 2,4-D in combination with Kn was used to induce callus and growth of callus was also studied. Four different concentrations of 2,4-D (0.5, 1.0, 2.0 and 3.0 mg/l) and 5 different concentrations of Kn (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l) were used.

Results obtained on callus induction, nature of callus, fresh and dry weights of callus are given in Table 33.

Remarkable effect of 2,4-D with Kn was observed in all the culture media in this experiment. Two mg/l of 2,4-D had the highest effect on callus induction compared to other concentrations of 2,4-D used. The highest of 66.67 percent of cultures induced callus when cultured on media containing 2.0 mg/l 2,4-D + 1.0 mg/l Kn (Plate–IV, C). The lowest percentage (46.67%) of cultures induced callus on media with 3.0 mg/l 2,4-D + 3.0 mg/l Kn, 0.5 mg/l 2, 4-D + 0.1 mg/l Kn, 0.5 mg/l 2,4-D + 0.5 mg/l Kn, 2.0 mg/l 2,4-D + 3.0 mg/l Kn and 1.0 mg/l 2,4-D + 3.0 mg/l Kn. It was noted that 2,4-D with Kn enhanced callus induction. There were considerable differences between the concentrations of 2, 4-D as well as within a 2,4-D concentration between Kn concentrations used on callus induction.

Table 33 : Effect of different concentrations of 2, 4 – D with Kn on callus induction from in vitro grown leaf segments of R. serpentina.

Growth regulators (mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2, 4 – D + Kn				
0.5+0.1	46.67	2.71	0.13	Greenish
0.5+0.5	46.67	2.75	0.13	Greenish
0.5+1.0	53.33	2.98	0.14	Greenish
0.5+2.0	60.00	3.00	0.14	Greenish
0.5+3.0	53.33	3.46	0.17	Greenish
1.0+0.1	53.33	3.00	0.14	Greenish
1.0+0.5	53.33	3.01	0.14	Greenish
1.0+1.0	60.00	3.51	0.17	Greenish
1.0+2.0	60.67	3.60	0.18	Greenish
1.0+3.0	46.67	3.02	0.15	Greenish
2.0+0.1	53.33	3.08	0.15	Greenish
2.0+0.5	60.00	3.05	0.15	Greenish
2.0+1.0	66.67	3.70	0.18	Greenish
2.0+2.0	60.00	3.60	0.18	Greenish
2.0+3.0	46.67	3.48	0.17	Greenish
3.0+0.1	53.33	3.12	0.15	Greenish
3.0+0.5	60.00	3.55	0.17	Greenish
3.0+1.0	53.33	3.51	0.17	Greenish
3.0+2.0	46.67	3.45	0.16	Greenish
3.0+3.0	46.67	3.12	0.15	Greenish
$LSD_{5\%}$	12.5	0.8	NS	

2,4-D concentration was also significantly effective on fresh weight of callus in culture media. Average callus weight was highest in medium containing 2.0 mg/l 2,4-D with all the concentrations of Kn. The highest callus weight (3.70 g/culture) was obtained from culture medium having 2.0 mg/l 2,4-D + 1.0 mg/l Kn.

The highest dry weight (0.18 g/culture) of callus was obtained in medium having 2.0 mg/l 2,4-D + 1.0 mg/l Kn. The lowest dry weight was obtained when 3.0 mg/l of 2,4-D was used with all the 5 concentrations of Kn. Marked differences in callus weight was observed between 2,4-D concentrations as well as among Kn concentrations within a 2,4-D concentration used on callus induction.

The nature of callus obtained was greenish and hard in texture.

Effect of Different Concentrations of 2,4-D with BA and CH on Callus Induction from *in vitro* Grown Leaf Segment

In this experiment 2, 4-D in combination with BA and CH was used to induce callus and callus growth was studied. Two mg/l 2,4-D was used with 4 different concentrations of BA (0.1, 0.5, 1.0 and 2.0 mg/l) as well as 5 different concentrations of CH (0.1,0.5, 1.0, 2.0 and 3.0 mg/l).

Results obtained on callus induction, nature of callus, fresh and dry weights of callus are tabulated in Table 34.

Marked effect on 2, 4-D with BA and CH was observed in all the couture media in this experiment. However, 0.5 mg/l BA and 1.0 mg/l CH with 2.0 mg/l 2,4-D had the highest effect on callus induction compared to other concentrations of 2,4-D used (Plate–IV, D). Percentage of cultures induced callus was 68.75% when

Table 34 : Effect of different concentrations of 2,4–D with BA and CH on callus induction from *in vitro* grown leaf segments of *R. serpentina.*

Growth regulators (mg/l)	% of callus induction	Average wt.of shoots/ explant (g) after 60 days		Type of callus
		Fresh wt.	Dry wt.	
2,4–D + BA + CH				
2.0+0.1+0.1	43.75	2.78	0.14	Greenish
2.0+0.1+0.5	43.75	2.81	0.14	Greenish
2.0+0.1+1.0	62.50	3.58	0.18	Greenish
2.0+0.1+2.0	56.25	3.51	0.17	Greenish
2.0+0.1+3.0	50.00	3.01	0.15	Greenish
2.0+0.5+0.1	43.75	3.46	0.17	Greenish
2.0+0.5+0.5	50.00	3.46	0.17	Greenish
2.0+0.5+1.0	68.75	3.80	0.19	Greenish
2.0+0.5+2.0	62.50	3.49	0.17	Greenish
2.0+0.5+3.0	56.25	3.48	0.17	Greenish
2.0+1.0+0.1	50.00	2.98	0.15	Greenish
2.0+1.0+0.5	56.25	3.58	0.18	Greenish
2.0+1.0+1.0	62.50	3.69	0.18	Greenish
2.0+1.0+2.0	62.50	3.70	0.18	Greenish
2.0+1.0+3.0	50.00	3.00	0.15	Greenish
2.0+2.0+0.1	56.25	3.69	0.18	Greenish
2.0+2.0+0.5	56.25	3.74	0.19	Greenish
2.0+2.0+1.0	48.75	3.38	0.17	Greenish
2.0+2.0+2.0	43.75	3.01	0.15	Greenish
2.0+2.0+3.0	37.50	3.00	0.15	Greenish
$LSD_{5\%}$	14.2	0.5	NS	

cultured on medium having 2.0 mg/l 2, 4-D + 0.5 mg/l BA and 1.0 mg/l CH. But minimum of 37.50 percent of cultures induced callus on medium containing 2.0 mg/l 2,4-D + 2.0 mg/l BA + 3.0 mg/l CH. Data revealed significant difference between the concentrations of BA used for callus induction. Significant difference was also noted within a BA concentration between CH concentrations.

Fresh weight of callus also showed remarkable effect of 2,4-D with BA concentration used in culture media. Average callus weight was highest when cultured on media having 2.0 mg/l 2,4-D and 0.5 mg/l BA with all the 5 concentrations of CH. On the other hand, the lowest callus weight was obtained in the media containing 2.0 mg/l 2,4-D + 0.1 mg/l BA with all the 5 concentrations of CH used. The highest fresh weight of callus was 3.80 g obtained in the media having 2,0 mg/l 2, 4-D + 0.5 mg/l BA + 0.5 mg/l CH. Results revealed that on average, 0.5 mg/l BA and 1.0 mg/l CH had significantly greater callus weight compared to other concentrations of BA and CH used.

The dry weight (0.19 g/l culture) of callus was highest in 2.0 mg/l 2, 4-D + 0.5 /l BA and it ranged from 0.14 g to 0.18 g. The lowest dry weight of callus was obtained on culture where 2.0 mg/l 2, 4-D + 0.1 mg/l BA was used with all the 5 concentrations of CH.

The nature of callus as obtained was greenish and hard in texture.

ROOT INDUCTION FROM *IN VITRO* GROWN SHOOT TIP IN DIFFERENT COMBINATIONS AND CONCENTRATIONS OF AUXINS

In vitro rooting is an important aspect of shoots developed through multiple shooting from embryogenic or organogenic callus for complete plantlet development. This part includes 4 experiments dealing with various aspects of *in vitro* rooting on micropropagated shoots. Auxins like NAA, IAA and IBA were used to induce roots. Data on percentages of cultures produced roots, number of roots per shoot, length of the longest root and fresh and dry weights of roots were recorded, analyzed and described.

Effect of Different Concentrations of Auxins on Root Induction from *in vitro* Grown Shoot Tip

This experiment was conducted to study the effect of three different auxins IAA, IBA and NAA on induction of roots of *in vitro* grown shoot tips collected from second sub cultured shoots. Five different concentrations (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of NAA and 5 different concentrations (2.0, 3.0, 4.0, 5.0 and 6.0 mg/l) of IAA and IBA were used.

Data on percentage of cultures induced roots were collected after 20 days of culture and are presented in Table 35. There was significant effect of three different auxins in respect of percentage of cultures induced roots. NAA was found to be more effective than IAA and IBA. IBA showed least response.

Table 35 : Effect of different concentrations of auxins on root induction from *in vitro* grown shoot tip of *R. serpentina.*

Growth regulators (mg/l)	% of root induction	Average no. of roots/explant after days			Average length of longest roots (cm) after 60 days	Average wt. of roots/explant(g) after 60 days	
		20	40	60		Fresh wt.	Dry wt.
IAA							
2.0	14.28	1.0	1.0	1.0	2.8	2.46	0.23
3.0	28.57	1.3	2.0	2.6	2.8	2.88	0.28
4.0	57.14	1.2	2.5	2.5	2.9	2.76	0.27
5.0	42.85	1.2	1.2	2.0	2.7	2.78	0.27
6.0	21.42	1.0	1.0	2.0	2.5	2.70	0.25
$LSD_{5\%}$	12.5	NS	0.5	0.6	NS	NS	NS
IBA							
2.0	7.14	1.5	2.0	2.5	2.7	2.13	0.20
3.0	21.42	2.0	2.0	2.6	2.8	2.34	2.23
4.0	50.00	2.5	3.0	3.1	2.9	2.93	0.28
5.0	35.71	2.3	2.3	2.6	2.7	2.52	0.24
6.0	14.28	1.5	2.5	2.6	2.4	2.12	0.20
$LSD_{5\%}$	16.3	0.6	0.8	NS	NS	NS	NS
NAA							
0.5	28.57	3.3	3.5	3.7	3.5	3.26	0.30
1.0	78.57	4.2	4.3	4.6	4.0	3.98	0.39
1.5	71.42	4.1	4.2	4.4	4.1	3.91	0.37
2.0	64.28	3.1	3.3	3.5	3.8	3.71	0.35
3.0	35.71	2.1	2.5	2.7	3.1	3.67	0.34
$LSD_{5\%}$	22.5	1.5	1.2	0.8	0.6	NS	NS

The ranges of percentages of cultures produced roots in different concentrations of NAA, IAA and IBA were 28.57% - 78.57%, 14.28% - 57.14% and 7.14%-50.00%, respectively. In NAA supplemented medium highest percentage (78.57%) of cultures induced roots was recorded in 1.0 mg/l and it was 78.57% whereas in IAA and IBA supplemented media highest percentage of cultures induced roots was observed in 4.0 mg/l of each the two auxins and the percentages were 57.14 and 50.00% respectively.

The minimum concentrations of NAA, IAA and IBA in culture media required to induce roots were 0.5 mg/l, 2.0 mg/l and 2.0 mg/l, respectively. Higher concentrations of these auxins significantly reduced the percentage of cultures induced roots. Average numbers of roots per explant as induced by the three types of auxins are shown in Table 35. Significant effect of auxins was noted among the 3 types of auxins used. Maximum of 4.6 roots per culture was recorded in NAA supplemented medium where 1.0 mg/l of auxin was used. The highest number of roots per explant was recorded as 2.6 and 3.1 per culture respectively in 3.0 mg/l IAA and 4.0 mg/l IBA (Plate – V, A). Most of the roots were induced within 20 days of culture.

The average length of the longest root was recorded and presented in Table 35. Auxin NAA was found to have significantly greater effect on root length compared to IAA and IBA. Root length as long as 4.1 cm was obtained in 1.5 mg/l NAA whereas in IAA and IBA the longest roots were always less than 3 cm.

Fresh weight of roots per culture was recorded after 60 days of culture and is presented in Table 35. The effect of different types of auxins was found and NAA showed greater root yield compared to IAA and IBA. The fresh weight of roots ranged from 3.26–3.98 g in NAA supplemented medium and in IAA and IBA

Plate V

A. Root formation in medium having MS + 1.0 mg/l NAA from shoot tip.

B. Root formation in medium having MS + 3.0 mg/l NAA+ 0.5 mg/l IAA from shoot tip.

C. Root formation in medium having MS + 1.0 mg/l NAA from nodal segment.

D. Root formation in medium having MS + 3.0 mg/l IBA + 2.0 mg/l IAA from nodal segment..

supplemented media the ranges were 2.46 – 2.88 g and 2.12 – 2.93 g, respectively. The highest fresh weight of roots per culture was recorded in 1.0 mg/l NAA which was 3.98 g.

After taking fresh weight of roots, roots were dried at 55°C in a temperature-controlled dryer for 5 days. A dry weight of roots per culture was also recorded after 60 days of culture and is presented in Table 35. The dry weight of roots between different concentrations within a type of hormone was found to be more or less same. The highest dry weight was recorded in 1.0 mg/l NAA medium and it was 0.39 g.

Effect of Different Concentrations and Combinations of IBA and IAA on Root Induction from *in vitro* grown shoot tip

Effect of IBA and IAA on root induction from *in vitro* grown shoot tips was studied. The different concentrations of IBA were 1.0, 2.0, 3.0 and 4.0 mg/l and each of the concentrations was combinedly used with different concentrations (0.5, 1.0, 2.0, 3.0 and 4.0) of IAA.

Data collected on percentage of cultures induced roots are shown in Table 36. Explants induced roots in all the concentrations of IBA with various concentrations of IAA. Explants failed to induce any root when the concentration of IBA was used below. 1.0 mg/l with any concentration of IAA.

Root induction frequencies were significantly different among different concentrations of IBA used. Best root induction was noted in 3.0 mg/l IBA with 0.5 – 3.0 mg/l IAA compared to other media composition. Higher concentration of IBA with any concentration of IAA induced reduced number of root. Maximum 62% of

the cultures induced roots when 3.0 mg/l IBA with either 0.5 or 1.0 mg/l IAA was used.

Average number of shoots per culture was recorded and are tabulated in Table 36. There was significant effect of combined action of the types of hormones. The root number per culture was high when 3.0 mg/l IBA with different concentrations of IAA was used in the culture media (Plate-V, B). The number of roots per culture increased with the increase in concentration of IBA with any concentration of IAA up to certain limit (3.0 mg/l IBA) but further increase in IBA concentration reduced the number of roots.

Length of the longest root was recorded and tabulated in Table 36. The length of the roots was higher when cultured on medium having 3.0 mg/l IBA with any concentration of IAA. In this medium concentration the root length was 3.0 to 3.6 cm but in other media the root length was always less than 3 cm.

Fresh weights of roots recorded from various media composition are shown in Table 36. The fresh weight was also influenced by the various concentrations of IBA. The fresh weight was significantly high in 3.0 mg/l IBA with different concentrations of IAA compared to other concentrations of IBA used in this experiment. The highest fresh weight of 3.43 g per culture was recorded in medium where 3.0 mg/l IBA + 0.5 mg/l IAA was used.

Dry weight of root was recorded in Table 36. The dry weight depended on different concentrations of both IBA and also IAA. The highest dry weight of roots per culture was recorded on media where 3.0 mg/l IBA was used with 0.5 to 2.0 mg/l IAA and it ranged from 0.31 to 0.33 g per culture. The lowest dry weight was recorded when 4.0 mg/l IBA was used with 3.0 mg/l and 4.0 mg/l of IAA.

Table 36 : Effect of different concentrations of IBA and IAA on root induction from *in vitro* grown shoot tip of *R. serpentina.*

Growth regulators (mg/l)	% of root induction	Average no. of roots/explant after days			Average length of longest roots (cm) after 60 days	Average wt. of roots/explant(g) after 60 days	
		20	**40**	**60**		Fresh wt.	Dry wt.
IBA+IAA							
1.0+0.5	12.50	1.0	1.0	1.0	2.5	2.31	0.22
1.0+1.0	18.75	1.7	2.1	2.1	2.6	2.46	0.23
1.0+2.0	31.25	1.8	2.0	2.2	2.7	2.51	0.24
1.0+3.0	25.00	1.5	1.7	1.9	2.2	2.39	0.22
1.0+4.0	18.75	1.0	1.3	1.6	2.1	2.16	0.20
2.0+0.5	18.75	1.0	1.5	1.5	2.1	2.48	0.23
2.0+1.0	25.00	2.0	2.0	2.0	2.6	2.65	0.25
2.0+2.0	25.00	2.3	2.3	2.6	2.7	2.76	0.26
2.0+3.0	37.50	2.5	2.7	2.9	2.9	2.94	0.28
2.0+4.0	31.25	2.5	2.6	2.7	2.8	2.71	0.25
3.0+0.5	62.50	2.8	3.0	3.7	3.5	3.43	0.33
3.0+1.0	62.50	2.5	2.7	2.9	3.2	3.33	0.32
3.0+2.0	56.25	2.3	2.5	2.8	3.0	3.31	0.31
3.0+3.0	50.00	2.3	2.5	2.9	3.1	2.79	0.26
3.0+4.0	12.50	2.1	2.3	2.5	2.9	2.60	0.24
4.0+0.5	50.00	2.2	2.5	2.7	2.8	2.38	0.22
4.0+1.0	43.75	2.0	2.1	2.5	2.6	2.44	0.23
4.0+2.0	31.25	1.8	1.8	2.0	2.3	2.17	0.20
4.0+3.0	25.00	1.6	1.7	2.0	2.1	2.09	0.19
4.0+4.0	12.50	1.0	1.0	1.0	1.9	2.05	0.19
$LSD_{5\%}$	22.6	0.5	0.6	0.5	NS	0.68	0.10

Effect of Different Concentrations of Auxins on Root Induction from *in vitro* Grown Nodal Segment

Nodal explants having one axillary bud were cultured to induce roots in NAA, IAA and IBA. The concentrations of auxins were 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l of NAA, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/l of IAA and 2.0, 3.0, 4.0, 5.0 and 6.0 mg/l of IBA.

Data obtained on percentage of cultures induced roots are shown in Table 37. NAA was found to induce roots in greater number of cultures compared to IAA and IBA. The percentage of culture that induced roots in different auxins was significantly different. The highest percentage of cultures induced root in NAA supplemented media and the lowest percentages of culture induced roots in IBA supplemented media. As many as 63.2% of cultures induced roots when 1.0 mg/l or 1.5 mg/l NAA was used. In IAA supplemented medium 50% of the cultures induced roots when 4.0 mg/l of IAA was added in the media. In IBA supplemented media only 42.85% of cultures induced roots in 4.0 mg/l of IBA in the culture. No root induction was observed when IBA concentration was below 1 mg/l with any concentration of IAA.

Average numbers of roots per culture was recorded and are tabulated in Table 37. The highest number of roots per culture was induced in NAA supplemented medium and it was 2.8 per culture in 1.0 mg/l NAA (Plate–V, C). In IAA supplemented media, the highest number of roots per explant was 2.2 when 4.0 mg/l IAA was added in the culture media. For IBA supplemented media 4.0 mg/l induced highest number of roots per culture, which was 2.4 roots per culture.

Table 37. Effect of different concentrations of auxins on root induction from *in vitro* grown nodal segment of *R. serpentina.*

Growth regulators (mg/l)	% of root induction	Average no. of roots/explant after days			Average length of longest roots (cm) after 60 days	Average wt. of roots/explant(g) after 60 days	
		20	40	60		Fresh wt.	Dry wt.
IAA							
2.0	14.28	1.6	1.8	2.0	2.7	2.28	0.21
3.0	21.42	1.5	1.7	2.0	2.9	2.36	0.22
4.0	50.00	1.8	1.9	2.2	2.8	2.46	0.23
5.0	35.71	1.7	1.8	2.1	2.5	2.27	0.21
6.0	14.28	1.5	1.5	1.9	2.4	2.18	0.20
$LSD_{5\%}$	13.5	NS	NS	NS	NS	NS	NS
IBA							
2.0	7.14	1.7	1.8	2.1	2.6	1.96	0.19
3.0	21.42	1.8	2.0	2.2	2.6	2.42	0.24
4.0	42.85	2.0	2.2	2.4	2.7	2.63	0.25
5.0	35.71	1.6	1.8	1.9	2.6	2.11	0.20
6.0	14.28	1.5	1.6	1.8	2.5	1.88	0.18
$LSD_{5\%}$	15.5	NS	NS	NS	NS	0.20	NS
NAA							
0.5	21.42	2.2	2.5	2.6	2.8	2.76	0.26
1.0	64.28	2.3	2.5	2.8	3.0	2.94	0.30
1.5	64.28	2.2	2.3	2.7	2.9	2.77	0.28
2.0	35.71	2.0	2.2	2.5	2.8	2.75	0.27
3.0	14.28	1.0	1.5	1.5	2.5	1.81	0.18
$LSD_{5\%}$	25.2	0.8	0.6	0.8	NS	0.35	0.09

Average length of the longest root was recorded at 60 days of culture and are shown in Table 37. The range of root length was 2.5 cm to 3 cm in NAA supplemented media, 2.4–2.9 cm in IAA supplemented media and 2.5–2.7 cm in IBA supplemented media.

Fresh weight of the roots are shown in Table 37. The highest fresh weight was recorded to be 2.94 g/culture in 1.0 mg/l NAA. In IAA and IBA supplemented media, the highest fresh weight was recorded to be 2.46 and 2.63 g respectively in 4.0 mg/l of the auxins used.

Dry weight of the root was significantly greater in NAA supplemented media compared to IAA and IBA supplemented media. The highest dry weight per culture was 0.30 g in 1.0 mg/ l NAA supplemented medium.

Effect of Different Concentrations and Combinations of IBA and IAA on Root Induction from *in vitro* Grown Nodal Segment

The effect of combined action of two types of auxins on root induction from nodal explants was evaluated in this experiment. Four concentrations of IBA in combination with 5 concentrations of IAA were used in this experiment. The concentrations of IBA were 1.0, 2.0, 3.0 and 4.0 mg/l. Each of the IBA concentrations was combinedly used with each of five concentrations of IAA (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l).

Data collected on percentages of cultures induced roots are shown in Table 38. The highest percentages of cultures induced roots were noted when 3.0 mg/l IBA

was used in the culture media. The range of variation in root induction in 3.0 mg/l IBA with different concentrations of IAA was 53.84% in 0.5 mg/l IAA to 15.38% in 4.0 mg/l IAA.

The data on number of roots per explant was collected and are shown in Table 38. The number of roots per culture was greater in 3.0 mg/l IBA compared to other concentrations. Reduced number of roots per culture among various concentration of IBA was noted. The highest number of roots was found in 3.0 mg/l IBA + 2.0 mg/l IAA (Plate–V, D).

Average length of roots per explant was measured at 60 days of culture. Average length was almost same in 1.0–3.0 mg/l IBA whereas least length was recorded when cultured on media having 4.0 mg/l IBA with all the concentrations of IAA. Root length of 3 cm per culture was noted in media where 3.0 mg/l IBA + 1.0 mg/l IAA was used. Moreover, no significant difference among the concentrations of IAA within a given concentration of IBA was noted.

Fresh weights of roots per culture were recorded at 60 days of culture and are shown in Table 38. There was a difference in fresh weight among the different concentrations of IBA used. Greater fresh weight was noted in 3.0 mg/l IBA. But less difference was observed in fresh weight among the different concentrations of IAA with a given concentration of IBA. Fresh weight was highest in 3.0 mg/l IBA + 2.0 mg/l IAA and it was 2.96 g/culture.

Greater dry weight of roots was found in 3.0 mg/l IBA compared to other concentrations of IBA used. Less difference in dry weight of roots was noted among the various concentrations of IAA used within a concentration of IBA. The highest dry weight of 0.28 g/culture was noted in 3.0 mg/l IBA with 1.0–2.0 mg/l of IAA concentration in the culture media.

Table 38 : Effect of different concentrations and combinations of IBA and IAA on root induction from *in vitro* grown nodal segment of *R. serpentina.*

Growth regulators (mg/l)	% of root induction	Average no. of roots/explant after days			Average length of longest roots (cm) after 60 days	Average wt. of roots/explant(g) after 60 days	
		20	40	60		Fresh wt.	Dry wt.
IBA+IAA							
1.0+0.5	7.69	1.0	1.0	1.0	2.5	2.28	0.21
1.0+1.0	15.38	1.5	1.7	2.0	2.6	2.30	0.22
1.0+2.0	30.76	1.8	2.0	2.3	2.8	2.40	0.23
1.0+3.0	23.07	1.7	2.0	2.2	2.6	2.10	0.20
1.0+4.0	15.38	1.5	1.8	2.0	2.5	1.96	0.18
2.0+0.5	15.38	1.2	1.3	1.8	2.6	2.33	0.22
2.0+1.0	15.38	1.8	2.0	2.4	2.7	2.18	0.20
2.0+2.0	23.07	2.1	2.4	2.6	2.7	2.38	0.22
2.0+3.0	38.46	2.3	2.5	2.7	2.8	2.24	0.22
2.0+4.0	30.76	2.1	2.3	2.5	2.4	2.26	0.21
3.0+0.5	53.84	2.0	2.2	2.5	2.5	2.70	0.26
3.0+1.0	46.15	2.5	2.4	2.6	3.0	2.96	0.28
3.0+2.0	38.46	2.5	2.6	2.8	2.9	2.82	0.28
3.0+3.0	23.07	2.2	2.5	2.7	2.8	2.19	0.23
3.0+4.0	15.38	1.8	1.9	2.5	2.6	2.13	0.13
4.0+0.5	46.15	2.0	2.1	2.3	2.3	2.37	0.23
4.0+1.0	38.46	2.1	2.3	2.5	2.4	2.35	0.23
4.0+2.0	30.76	2.0	2.2	2.4	2.5	2.21	0.20
4.0+3.0	15.38	1.6	1.7	2.0	2.4	2.05	0.19
4.0+4.0	7.69	1.0	1.2	1.6	2.2	1.81	0.17
$LSD_{5\%}$	20.5	0.8	0.6	0.6	NS	0.16	NS

Plate VI

Potted plantlet of *R. serpentina* after 8 weeks of transplantation to the soil

COMPARISON OF RESERPINE CONTENT IN DIFFERENT PLANT PARTS

Percentages of reserpine alkaloid presents in the various plant parts of field grown as well *in vitro* grown shoots, roots and leaves were estimated. Estimation of reserpine obtained from various plant parts are shown in Tables 39, 40 and 41. Reserpine content was found to be 0.192% and 0.200% respectively in field grown and *in vitro* grown roots. In shoots excluding roots, the reserpine percentages were 0.06% and 0.065% in field grown and *in vitro* grown plants, respectively (Table 39).

In case of leaves the percentages of reserpine were 0.050 and 0.052%, respectively in field grown and *in vitro* grown plants. It was noted that *in vitro* grown plant parts possessed greater amount of reserpine compared to those in field grown plant parts (Table 39).

Table 39 : Reserpine present in different parts of *R. serpentina* plant.

Source of plant	Name of the plant parts	% of reserpine
Field grown	Root	0.192±0.010
In vitro grown	Root	0.220±0.011
Field grown	Shoot	0.060±0.008
In vitro grown	Shoot	0.065±0.007
Field grown	Leaf	0.050±0.004
In vitro grown	Leaf	0.052±0.005

In the second phase of reserpine estimation it was considered the hormonal level in the media. The materials were collected from medium containing 1.0 mg/l

NAA, 3.0 mg/l IBA + 0.5 mg/l IAA, 2.0 mg/l BA + 0.2 mg/l NAA, 2.0 mg/l 2, 4-D + 0.5 mg/l BA and 2.0 mg/l 2.4-D + 0.5 mg/l BA + 0.1 mg/l CH. Plant parts like root, shoot, leaf, calli were collected and estimation of reserpine was made. It was also determined the presence of reserpine in cultured media with an assumption that some alkaloids might transfer from plants to the culture medium.

In NAA supplemented media, reserpine content was highest in the root and lowest in the media. It was 0.200, 0.058, 0.053 and 0.040% respectively in root, shoot, leaf and the media. Almost 4 times reserpine were found to be present in the roots compared to shoot and leaves. Media also possessed some reserpine (Table 40).

The percentage of reserpine that was estimated for root, shoot, leaf and media as obtained from 3.0 mg/l IBA + 0.5 mg/l IAA are shown in Table 40. The roots possessed the highest percentage of reserpine which was 0.15%. Reserpine percentage in shoot and leaf were 0.06 and 0.05, respectively. In the media, 0.04% of reserpine was estimated. It was noted that roots induced in medium supplemented with NAA possessed higher alkaloid percentage compared to IBA + IAA supplemented media (Table 40).

In the medium containing 2.0 mg/l BA + 0.2 mg/l NAA, shoot, leaf and base callus were tested for reserpine production. The amount of reserpine was 0.058, 0.052 and 0.038% respectively in shoot, leaf and base callus. The culture media contained only 0.04% reserpine (Table 40). The percentages of reserpine were 0.10 and 0.11% respectively in leaf and internode derived calli induced in 2.0 mg/l 2,4-D + 0.5 mg/l BA. This reserpine was found to induce in both leaf and internode callus when 0.1 mg/l CH was added to 2.0 mg/l 2.4-D + 0.5 mg/l BA (Table 40).

Table 40 : Reserpine present in different media composition.

Media composition (mg/l)	Name of the plant parts	% of reserpine
1 NAA	Root	0.20 ± 0.009
1 NAA	Shoot	0.058 ± 0.006
1 NAA	Leaf	0.053 ± 0.004
1 NAA	Media	0.040 ± 0.007
3 IBA + 0.5 IAA	Root	0.150 ±0.012
3 IBA + 0.5 IAA	Shoot	0.060 ± 0.007
3 IBA + 0.5 IAA	Leaf	0.050 ± 0.009
3 IBA + 0.5 IAA	Media	0.040 ± 0.007
2 BA + 0.2 NAA	Shoot	0.058 ± 0.004
2 BA + 0.2 NAA	Leaf	0.052 ± 0.008
2 BA + 0.2 NAA	Media	0.041 ± 0.009
2 BA + 0.2 NAA	Base callus	0.038 ± 0.006
2 2, 4 – D + 0.5 BA	Leaf callus	0.100 ± 0.007
2 2, 4 – D + 0.5 BA	Internode callus	0.110 ± 0.007
2 2, 4 – D + 0.5 BA	Media	0.040 ± 0.009
2 2, 4 – D + 0.5 BA + 0. 1 CH	Leaf callus	0.105 ± 0.006
2 2, 4 – D + 0.5 BA + 0. 1 CH	Internode callus	0.113 ± 0.007
2 2, 4 – D + 0.5 BA + 0. 1 CH	Media	0.040 ± 0.004

Table 41 : Effect of different pH adjusting agents on reserpine production in NAA supplemented media.

Media composition (mg/l)	Level of pH	Name of the acid/alkali	Name of the plant parts	Percentage of reserpine
1 NAA	3.0	HCl	Root	0.20 ± 0.007
1 NAA	3.5	HCl	Root	0.20 ± 0.006
1 NAA	4.0	HCl	Root	0.20 ± 0.009
1 NAA	4.5	HCl	Root	0.20 ± 0.007
1 NAA	5.0	HCl	Root	0.20 ± 0.008
1 NAA	5.8	$Ca(OH)_2$	Root	0.21 ± 0.011
1 NAA	5.8	KOH	Root	0.20 ± 0.014
1 NAA	5.8	NaOH	Root	0.20 ± 0.011
1 NAA	6.0	$Ca(OH)_2$	Root	0.215 ± 0.002
1 NAA	6.0	KOH	Root	0.200 ± 0.009
1 NAA	6.0	NaOH	Root	0.200 ± 0.011
1 NAA	6.5	$Ca(OH)_2$	Root	0.217 ± 0.002
1 NAA	6.5	KOH	Root	0.200 ± 0.009
1 NAA	6.5	NaOH	Root	0.200 ± 0.007
1 NAA	7.0	$Ca(OH)_2$	Root	0.220 ± 0.004
1 NAA	7.0	KOH	Root	0.200 ±0.005
1 NAA	7.0	NaOH	Root	0.200 ± 0.006
1 NAA	7.5	$Ca(OH)_2$	Root	0.210 ± 0.009
1 NAA	7.5	KOH	Root	0.200 ± 0.012
1 NAA	7.5	NaOH	Root	0.200 ± 0.014
1 NAA	8.0	$Ca(OH)_2$	Root	0.208 ± 0.011
1 NAA	8.0	KOH	Root	0.198 ± 0.014
1 NAA	8.0	NaOH	Root	0.195 ± 0.012
1 NAA	8.5	$Ca(OH)_2$	Root	0.200 ± 0.011
1 NAA	8.5	KOH	Root	0.195 ± 0.009
1 NAA	8.5	NaOH	Root	0.193 ± 0.011

In another experiment, the effects of various pH levels on reserpine production was tested. In this case only roots were used. For this pH was adjusted from 3.0–8.5 using HCl, $Ca(OH)_2$, KOH and NaOH. The results obtained are shown in Table 41.

Low pH level when adjusted with HCl (3-5) had no effect on reserpine content of root and it was found to be 0.2% in all cases. The highest pH from 5.8–8.5 were adjusted using $Ca(OH)_2$, KOH and NaOH. The reserpine content was always higher in root when pH was adjusted with $Ca(OH)_2$. The highest percentage of reserpine in roots was recorded at pH 7.0 level adjusted with $Ca(OH)_2$ and it was 0.22%. For KOH and NaOH reserpine percentages estimated were found to be the same in all the pH levels except in 8.0 and 8.5 pH where KOH gave slightly higher reserpine compared to NaOH.

DISCUSSION

Mass propagation of plant species *in vitro* is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for some medicinal plants. Tissue culture in propagation and its importance in conservation of genetic resources and clonal improvement has been described by different authors (Barz *et al.*, 1977; Datta and Datta, 1985; Kukreja *et al.*, 1989).

In vitro methods of plant propagation include shoot culture with proliferation of axiliary or adventitious shoots and callus culture with organogenesis or embryogenesis. Though all plant cells are theoretically totipotent, attempts with any

tissues to get whole plants lead to failure due to lack of proper techniques and insufficient knowledge about nutrient media and other physical and chemical conditions. which are essential for proper growth of cells, tissues and organs (Johri, 1982).

In any preliminary study on the tissue culture of plants, it is customary to use juvenile tissues rather than those from mature plants. This is because most plant populations are highly heterogeneous, the tissues are difficult to grow, and if they grow, they also show high variability in culture responses. Mature plants are also less responsive when compared with juvenile tissues (Sommer and Caldras, 1981). Moreover, juvenile plant parts regenerate adventitious shoots relatively easily. For example, with gymnosperms, adventitious shoot formation is often only possible with embryos, seedlings or parts of seedlings and not mature plants.

In the present study with *R. serpentina*, materials from young branches of field grown plants were used for primary establishment of cultures. For shoot proliferation, shoot tips and nodal explants from 8-9 months old field grown plants were used as explant sources. Explants from actively growing shoots at the beginning of the growing season generally give the best results for enhanced axillary branching (Yang, 1977). Hence, explants were taken from young, newly formed shoots of the plants. These explants were placed on media with different concentrations of cytokinins, with or without auxins. Cytokinins have been shown by many workers to release lateral buds from apical dominance (Rubinstein and Nagao, 1976).

Stimulation of axillary shoots from single nodes proved to be most prolific source of shoots for many varieties of citrus (Barlass and Skene, 1982). In this study, nodal explants as well as shoot tips were cultured on MS basal medium supplemented with different concentrations of BA (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mg /l).

Although exogenous auxins do not promote axillary shoot proliferation, culture growth may be improved by their presence. One of the advantages of adding auxins at low concentration to the culture media is to nullify the effect of the higher concentration of cytokinin on axillary shoot elongation (Hu and Wang, 1983).

In the present investigation, the callus formation at the basal portion of the nodal explants and shoot tips inhibited growth of the axillary buds in a high percentage of cultures. It was observed that the frequency of callus formation was greater in cultures of shoot tips than nodal explants. Formation of base callus significantly decreased the frequency of multiple shoot formation, number of shoots per explant and also shoot length. On average, more than 4 shoots from one explant were produced in media having 2.0 mg/l BA + 0.2 mg/ l NAA. This proliferation rate was maintained up to 10^{th} subculture. Some of the shoots grew rapidly and others grew slowly. However, the regenerated shoots reached to a height of about 5 cm if the cultures were maintained for 45 days. It was observed that increasing the BA concentration increased the percentage of explant produced shoots and the optimum concentration was 2.0 mg / l irrespective of concentrations of NAA used (Table 22). When more than 2.0 mg / l BA was used, the percentage of explant responded to multiple shooting decreased and at a certain stage the response was completely ceased (Table 22). This implies that high concentration of BA was deleterious to the explants. Dunstan *et al.* (1985) reported that although increasing exogenous BA induced axillary bud break, high concentration of BA could be detrimental to shoot quality and smaller and unusable shoots were produced. Yadav *et al.* (1990) and Zaman *et al.* (1992) in mulberry and Banu *et al.* (1997) in *Adhatoda vasica* also reported that 2.0-mg / l BA was more effective for shoot multiplication and elongation.

Using GA_3 with BA and Kn did not increase the number of shoots per explant like BA with NAA and Kn with NAA (Tables 23 & 25). Optimal concentration of GA_3 was 0.1 mg / l with 2.0 mg / l BA and 0.5 mg / l with Kn. However, in GA_3 supplemented medium, shoot enlarged and grew healthy with several well-developed leaves within 3–4 weeks of culture. This result contradicts with Smith and Kefferd (1964) who reported that GA_3 appeared to be responsible only for the breaking of dormancy. But in *Atriplex*, GA_3 was found to be effective not only in stimulating shoot elongation but also in enhancing shoot number.

The most effective factor for shoot multiplication from either lateral or apical meristem is auxin-cytokinin combination (Wochok and Sluis, 1980). In many cases, GA_3 is also added to the media. Boxus *et al.* (1977), however, reported that addition of GA_3 in culture medium inhibited shoot proliferation in strawberry. Bhojwani and Razdan (1983) suggested that the application of GA_3 promotes budding. The endogenous level of the hormone must be suboptimal.

The optimal BA concentration for shoot proliferation was studied carefully. In the initial culture media when BA was used singly; optimal concentration was found to be 3.0 mg / l, whereas in the subsequent subcultures comparatively low concentration of BA was beneficial. In pear plants, 2.2 mg / l proved to be the most effective concentration of BA for shoot tip culture (Lane, 1979). However, stem of *Musa* could be induced to form multiple shoots most effectively on media with high concentration (5.0 mg / l) of BA (Cronauer and Krikorian, 1984a,b).

Although some explants on a particular medium showed good response, others on the same medium did not respond in the same way. Three factors may be involved: physiological status of the explants, the size of the explants and quality of the plant from which the explants were obtained (Murashige, 1974; Hu and Wang,

1983). Although these parameters were kept constant while choosing the explants, it was not possible to ensure that all the explants were exactly similar. Collection of explants at different periods of the year could also bring about some variations in response.

Many of the problems of inducing callus from plant tissue may be over come by using parts of freshly germinated plantlets ensuring tissue fragments composed of cells with high potential (Yeoman and Forche, 1980). Only younger parts of the growing shoots were selected as explant source to induce callus and axillary shoots in this investigation.

Though all the plant cells are theoretically totipotent, attempts with many tissues to get whole plants lead to failure due to lack of proper techniques and insufficient knowledge about nutrient media and other physical and chemical conditions, which are essential for proper growth of cells, tissues and organs (Johri, 1982). Callus is an actively dividing non-organized tissues of undifferentiated and differentiated cells often developing from injury or in tissue culture (Pierik, 1987). An exogenous supply of growth hormones is often recommended to initiate callus from different explants. Proper combinations of auxin-cytokinin to the medium are essential for callus induction but their requirements depend strongly on the genotype and endogenous hormone content of explants (Pierik, 1987). Rao and Lee (1986) reported that intermediate levels of auxin and cytokinin usually promote callusing. But many other factors like, genotype, composition of the nutrient media, physical growth factors, such as light, temperature, moisture etc., are also important for callus initiation (Pierik, 1987).

Although major emphasis has been given on Angiosperm tissues, callus initiation has been observed in gymnosperms, ferns, mosses, and liverworts (Yeoman,

1970; Yeoman and Macleod, 1977). Recently, many reports are available on callus initiation from herbaceous and medicinal and vegetable crop plant species (Kamat and Rao, 1978; Fassuliotis *et al.*, 1981: Bhat and Fassuliotis, 1981; Matsuoka and Hinata, 1983; Misra *et al.*, 1983; Jain *et al.*, 1985; Nishio *et al.*, 1987; Saxena *et al.*, 1987; Sihachakr and Ducreux, 1987; Akram and Ilahi, 1985, 1986; Ilahi and Akram, 1987; Ilahi *et al.*, 1988; Sarker *et al.*, 1996; Roy *et al.*, 1994, 1995; Banu *et al.*, 1997).

The present study on callus induction was conducted with internodes and leaves of *R. serpentina* to observe their callus induction capacity. MS medium supplemented with different auxin-cytokinin combinations was used to study the callusing response. Two types of plant materials were used; field grown plants and *in vitro* grown plants. 2,4-D, NAA, IBA and IAA were used as auxins and BA and Kn were used as cytokinins in different concentrations. It was found that callus proliferation strictly depended on exogenous hormone supplementation. In absence of exagenous hormone, explants failed to induce callus and became necrotic and died within a few days.

Among the four auxins used, 2, 4-D was found to be the best in respect of callusing response. On the other hand, BA was superior to Kn for callus growth when supplemented with 2, 4-D (Table 27). Normally, lower concentrations of cytokinin in combination with higher concentration of an auxin was favourable for callus induction. There are well documents on callus proliferation when auxins were used alone or in combination with cytokinin (Rao *et al.*, 1981; Lee *et al.*, 1982; Hammerschlag *et al.*, 1985; Akram and Ilahi, 1985; 1986; Ripley and Preece, 1986; Jain *et al.*, 1988; Espinasse *et al.*, 1989; Niedz *et al.*, 1989; Verhagen and Wann, 1989; Roy and De, 1990; Banu *et al.*, 1997).

In the present investigation, it was observed that callus initiation started from the cut surfaces of the explants within 4 to 5 days of culture and the whole explants turned into callus masses within 12 to 15 days. Calli derived from the both explants (internode and leaves) were soft, but calli derived from internodes were white and those from leaves were green. When the auxins 2, 4-D, NAA, IBA and IAA were used separately, maximum 83.33% of the cultures responded to callusing in 3.0 mg / l 2,4-D, whereas 41.66% cultures responded to callus in 3.0 mg / l NAA, 4.0 mg / l IBA as well as 4.0 mg / l IAA. The highest fresh weight of callus (3. 64 g / culture) was obtained in 3.0 mg / l 2, 4-D (Table 27).When BA and Kn were added separately to 2, 4-D supplemented media, callus induction started within 2 to 3 days and the explant turned into callus masses within 8 to 10 days. One hundred percent of callus induction was observed from internode explants in 2.0 mg / l 2, 4-D+0.5 mg / l BA (Table 28). The callus weight was further increased to about 4. 54 g per culture when 0.1 mg / l CH (casein hydrolysate) was added to the medium (Table 30).

On the other hand, when leaf segments were cultured with different auxins, 66.66% of cultures responded to callus in 3.0 mg / l 2, 4-D and maximum fresh and dry weights of 2.69 and 0.12 g per culture, respectively was achieved. The percentage of culture and fresh and dry weights were further increased in 2.0 mg/ l 2, 4-D+0.5 mg/ l BA + 0.5 mg/ l CH (Table 30). Therefore, the combination of 2,4-D and BA was the most effective formulation for both explants used in the induction of callus and growth of callus, whereas IBA alone or either with BA or Kn was the least effective to induce callus formation. Oka and Ohyama (1976) got better results in medium with 2,4-D than IAA or NAA in mulberry which is in agreement with the present findings. Illahi and Akram (1987) also reported that 2,4-D used alone was considerably effective for callus formation in leaf segments of *R. serpentina* at various concentrations ranging from 0.1 to 1 mg/ l than NAA. These results are in

agreement with Staba (1969) who found 2,4-D alone as an effective auxin mostly at a concentration of 1 mg/ l.

Many workers observed 2,4-D as the best auxin for callus induction as common in monocot and even in dicot (Evans *et al.*, 1981; Lu *et al.*, 1982; Ho and Vaxil, 1983; Litz, 1984; Jaisal and Narayan, 1985; Chee, 1990). Kamat and Rao (1978) reported that 2,4-D which was most potent in stimulating the callus growth of explants proved in effective when used alone. This statement is also in agreement with the present findings. However, the present results differed with the work of steward *et al.*, (1958) who observed that elimination of 2,4-D from media induced callus proliferation in carrot cell cultures. This result also contradicts with the report of Medora *et al.*, (1979) who demonstrated inhibitory effect of BA in combination with 2, 4-D on induction and growth of callus in papaya.

Shoots obtained *in vitro* have to be rooted to obtain complete plants. In the present investigation, it was observed that field grown explants failed to induce root formation when placed in a culture medium. Hence, shoots obtained from *in vitro* grown plants were subcultured in MS basal medium supplemented with different concentrations of auxins, NAA, IBA and IAA alone and in combination. Shoots obtained from first and second subcultures failed to induce root formation. Rooting of *R. serpentina* started only when explants were taken from third subcultures. Most frequently, root formation is inhibited by the cytokinin used to induce shoot multiplication, so that the shoots do not produce roots *in vitro* until they were cultured on a medium containing auxins (George and Sherrington, 1984). Generally, rooting was induced within 10 to 15 days of transfer to root inducing medium for both explants (internodal segment having one axillary bud and shoot tips).

In the present experiment, NAA was superior to other auxins when used singly or combinedly with the other auxins for rooting. Among the different concentrations tested, 1.0 mg/l NAA was found to be the best concentration for root induction and growth of roots. The combination of IBA and IAA (3.0 mg/l IBA + 0.5 mg/l IAA) was more effective for rooting than any concentration of single IBA or IAA. One hundred percent of root cultures responded to rooting in 1.0 mg/l NAA when the explant source was shoot tips.

The percentage of cultures responded to rooting was always higher in shoot tip explants than nodal explants having one axillary bud. This result is similar to the result of Mathur *et al.* (1987) who reported that 1.5 mg/ l NAA was the best for root induction among all other auxins for *R. serpentina*. The number of roots per explant, the length of roots and fresh and dry weights of roots were higher in 1.0 mg/ l NAA. Roy *et al.* (1985) also reported that 1.0 mg/ l IBA + 1.0 mg/ l IAA was the best combination of auxins for proper rooting in *R. serpentina* and 100% shoots rooted within four weeks of culture. Roots began to emerge from tenth day of culture and within a period of 23–28 days 100% shoots rooted. Banu *et al.* (1997) observed that the average number of roots of *Adhatoda vasica* was better in NAA than those of IBA and IAA and even in NAA + IAA formulation. In 2.0 mg/ l NAA, the highest average number of roots was found to be 6.00.

The results of the present investigation clearly show that both the shoot tips and nodal explants of *R. serpentina* plant are capable of producing multiple shoots *in vitro* and subsequentljy root to form complete plantlets. After acclimatization, the plantlets were planted in the field where 80% of plants survived.

When all the steps of micropropagation of *R. serpentina* had been optimized, reserpine content in the different types of cultures was studied. During the study of

alkaloid content in the different parts (roots, leaves, stems) of *in vitro* grown plant, alkaloid content of field grown plant was also studied as a control. In order to know and compare the content of alkaloid in shoots and leaves of *in vitro* grown plants, the stem and leaves of field grown plants were also studied.

Roots of *R. serpentina* contain 0.15–0.20% reserpine–rescinnamine group of alkaloids (Anonymous, 1969). In the present study, root has been found to contain 0.192% reserpine in 8 – 9 months old field grown plants. The *in vitro* grown plant materials used for assay of reserpine content were collected from the cultures of the 4th subculture. It has been previously reported by Marta *et al.* (1992) that alkaloid production increased after 4th subculture. Alkaloids are accumulated in the cell when the growth of cells stopped (Marta *et al.*, 1992). Considering this idea, different plant parts were collected after 8 weeks of 4th subculture or subsequent subculture. After 8 weeks of any subculture, growth of roots was found to be decreased or stopped. Alkaloid content varied greatly among the different types and concentrations of phytohormones. Significantly greater amount (0.22%) of reserpine was obtained from the roots grown in the medium when NAA was supplemented than from IAA or IBA. Further increase in reserpine content was achieved while pH of the medium was raised to 6.0–7.0 by adjusting with $Ca(OH)_2$. The roots in this medium were hairy. Hairy roots were found to contain more alkaloid than smooth ones (Hamill *et al.*, 1986; Mano *et al.*, 1989; Verpoorte *et al.*, 1991 and Pyne *et al.*, 1987). Superiority of NAA over other auxins for the metabolites has also been reported by other investigators like Tabata *et al.* (1971) in tobacco, Tabata *et al.* (1974) in *Lithospernum erythrorhizon*, Zenk *et al.* (1975) in *Morinda citrofolia*. The alkaloid content of roots of media supplemented with other two auxins (IBA and NAA) was significantly low.

There was little amount (0.05%) of reserpine obtained from both *in vitro* and field grown shoots. Roja *et al.* (1980) also claimed that little amount of reserpine–like alkaloid, ajmalidine, was present in shoot culture. In the present study, the alkaloid has been obtained as reserpine. No variation was observed in the content of reserpine obtained from shoots cultured in different hormonal combinations.

In the present investigation, internode and leaf derived calli induced in medium supplemented with 2, 4-D, BA and CH were used. The amount of reserpine obtained from the calli ranged from 0.08–0.09%. Similar results were obtained by Heijden *et al.* (1989) in *Tabernaemontana elegans*. Illahi *et al.* (1988) analysed root, stem and leaf calli for *Rauwolfia* alkaloids. Ajmaline was the major alkaloid produced by the cultures. Alkaloids in such plants were higher in leaf and stem cultures than the parent plant.

The alkaloids produced by the *in vitro* grown plants may be secreted into the culture media where they were grown. Having this assumption, all the nutrient media from which plant parts were used for alkaloid extraction were also analyzed for the presence of reserpine. In all the cases reserpine was found to present in the culture media irrespective of type and concentration of phytohormones, but the amount was very low (0.03-0.04%). Yamamoto *et al.* (1987) also demonstrated the presence of alkaloid, berberine in tissue culture media of *Thalictrum minus*.

Now the question arises why does NAA supplemented media contain high amount of reserpine? When the pH of the medium was raised to 6-7 by adding Ca $(OH)_2$ to the medium, it further increased reserpine content. Dicosmo and Towers (1984) made an extensive review regarding the effect of different factors, like temperature, pH, nutrition (vitamins, minerals, etc) or phytohormones on secondary metabolite production in cultured plant cells.

In the present study, it was not possible to estimate the production of reserpine considering all the factors individually. However, it could be concluded that the auxin NAA had a stimulating effect in the reserpine production. Further, it may be assumed that at neutral pH range, any of the alkaloid biosynthetic enzymes is activated by Ca^{++}, which has wide activity on enzymatic level. Peroxidase enzymes have been reported to play a role in the biosynthesis of indole alkaloids (Smith *et al.*, 1988; Goodbody *et al.*, 1988a, b, c; Endo *et al.*, 1987; 1988). Ca^{++} has been found essential for maintaining the protein structure in the heme environment of the horseradish peroxidase (Ogawa *et al.*, 1979) and it has also been reported to have great influence on the peroxidase (Sticher *et al.*, 1981; Kevers *et al.*, 1982).

In tissue cultures, the known alkaloids are reported as altogether absent or when present are in traces. This behaviour is well reasoned and documented by various researchers (Luckner *et al.*, 1977; Hirotani and Furuya, 1977; Bauch and Leister, 1978). Although secondary metabolites may be produced in large quantities by cultures but the type of compound produced is often unexpected or even novel and specific compounds of the species might not be produced at all. Stockigt *et al.* (1981) were able to screen 10 previously not reported alkaloids from cell cultures of *R. serpentina.* The yield of vomilenine was 51 times higher in cultures than that found in differentiated plants.

This does not necessarily imply that best known plant products or particular secondary metabolites can only be produced by organized cell system but suggests that cells growing *in vitro* under arbitrary conditions do not always produce a characteristic secondary metabolite, particularly when reverted to a more basic mode of existence of replicative growth with conditions approaching favourable for growth of microorganisms.

However, new techniques are available to modify and improve the biosynthetic potential of cultured plant cells. In some plant species, cultures contain higher contents of secondary metabolites than intact plants e.g. pseudo-phedrine from *Ephedra foliata* is much higher (2.25%) in suspension than that in stem (Khanna *et al.*, 1976). Production of diosgenin in *Trigonella foenum-graecum* cultures was found at 1.82% by Khanna and Jain (1973) as compared to seeds containing 1.6%. By the use of 50 mg/100 ml cholesterol in culture media they were able to increase diosgenin content to 3.54%. Solasodine was increased up to a maximum of 680 µg/g dry weight with 70 mg/100 ml cholesterol in cultures of *Solanum xanthocarpum* (Khanna and Manot, 1976). Feeding of ascorbic acid to cultures of *Tagetes erecta* increased pyrethrin contents (Khanna and Khanna, 1976). Similarly, Khanna *et al.* (1978) were able to increase the *in vitro* production of major opium alkaloids with 12.5 mg/100 ml tyrosine. In *R. serpentina*, feeding experiments are not known except nutritional additives as coconut milk (Mitra and Kaul, 1964) or yeast extract (Ohta and Yatazawa, 1979) and casein hydrolysate (Ilahi *et al.*, 1988). Nevertheless, the cultures produced 96. 23^% more ajmaline in dark when compared to light grown cultures (Ilahi and Akram, 1987). Therefore, it is assumed that new alkaloids of *R. serpentina* cultures could be produced. This presumption is well supported by the work of Stockight *et al.* (1981) and Schubel and Stockight (1984).

Recently a rather new technique using enzyme systems for regulating biosynthetic potentials in *Rauwolfia* cultures is given by Stockight (1984) and Pfitzner *et al.* (1984).

With these techniques and improvements, plant tissue and cell culture systems could play an important role in the manufacture of biological compounds of therapeutic value.

SUMMARY

R. serpentina is vegetatively propagated by stem and root cuttings and has a poor seed viability and low germination percentage. Tissue culture methods for propagation offer an effective and quicker way to overcome the obstacles for production of a large number of propagules. Tissue culture techniques are becoming increasingly popular as an alternative means of plant vegetative propagation. Significant advantage is offered by the aseptic methods of clonal propagation over the conventional methods and a large number of plants can be produced from a single individual.

Tissue culture methods would be a valuable alternative for rapid propagation and conservation of this valuable threatened plant species.

In the present investigation various *in vitro* aspects of tissue culture have been tried with different explants of *R. serpentina*. Young branches of field grown plants were used for primary establishment of cultures. For shoot proliferation, shoot tips and nodal explants from 8-9 months old field grown plants were used as explant sources.

Callus formation at the basal portion of the nodal explants and shoot tips inhibited growth of the axillary buds in a high percentage of cultures. It was observed that the frequency of callus formation was greater in cultures of shoot tips than nodal explants. Formation of the base callus significantly decreased the frequency of multiple shoot formation, number of shoots per explant and also shoot length.

Increasing the BA concentration increased the percentage of explant produced shoots and the optimum concentration was 2.0 mg/ l irrespective of concentrations of NAA used.

Using GA_3 with BA and Kn did not increase the number of shoots per explant like BA with NAA and Kn with NAA. Optimal concentration of GA_3 was 0.1 mg/ l with 2.0 mg/ l BA and 0.5 mg/ l with Kn.

In the present investigation, MS medium supplemented with different auxin-cytokinin combinations was used to study the callusing response. Field grown plants and *in vitro* grown plants were used for this purpose. It was found that callus proliferation strictly depended on exogenous hormone supplementation. In absence of exogenous hormone, explants failed to induce callus and became necrotic and died within a few days.

Among the four auxins used, 2,4-D was found to be the best in respect of callusing response. On the other hand, BA was superior to Kn for callus growth when supplemented with 2,4-D.

The combination of 2,4-D and BA was the most effective formulation for both explants (internode and leaves) used in the induction of callus and growth of callus, whereas IBA alone or either with BA or Kn was the least effective to induce callus formation.

Field grown explants failed to induce root formation when placed in a culture medium. However, shoots obtained from the first and the second subcultures failed to induce root formation. Rooting took place only when explants were taken from third subcultures. NAA was superior to other auxins when used singly or combinedly with

the other auxins for rooting. Further, the percentage of cultures responded to rooting was always higher in shoot tip than nodal explants having one axillary bud.

The results clearly show that both the shoot tips and nodal explants of *R. serpentina* plant are capable of producing multiple shoots *in vitro* and subsequently root to form complete plantlets.

In order to know and compare reserpine content in shoots and leaves of *in vitro* grown plants, reserpine content of field grown plants were also studied as a control. It was noted that *in vitro* grown plant parts possessed greater amount of reserpine compared to those in field grown plant parts. Almost 4 times reserpine were found to be present in roots compared to shoot and leaves. Culture media also possessed some amount of reserpine.

CHAPTER 7

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APPENDIX I

Some meteorological data of North Bengal University experimental field (average of 1998, 1999 and 2000)

Month	Mean temperature (°C)	Rainfall (mm)	Relative humidity (%)
January	16.8	8.6	39
February	21.8	8.2	42
March	26.3	28.5	38
April	29.8	48.3	40
May	33.2	125.6	51
June	34.4	316.5	65
July	33.8	510.5	70
August	31.6	442.6	76
September	28.8	310.5	85
October	28.6	112.6	68
November	26.2	14.6	58
December	21.5	3.5	50