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Mukesh
Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Sharma SK
Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Corresponding Author:
Mukesh
Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Development of specific culture media, studies on effect of media, pH and temperature on growth of *Cercospora punicae*, the causal agent of fruit and leaf spot disease of pomegranate

Mukesh and Sharma SK

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Abstract
The mycelail growth of *Cercospora punicae* on PDA is very slow therefore the present investigation describes the effects of media, pH, temperature and standardized the best media for qucik growth of *Cercospora punicae* (leaf and fruit spot disease of pomegranate) *in vitro*. Five media, Potato Dextrose Agar (PDA), Czapeck Dox Agar (CDA), Green Pomegranate Leaf Decoction Agar 5% (GPLDA), Oat Meal Agar (OMA) and Vegetable Juice Agar (V-8JA) were evaluated for their effects on growth of the pathogen. Green Pomegranate Leaf Decoction Agar medium 5% (57.50 mm) was found to support maximum mycelail growth of fungus *in vitro*. Therefore, five different concentrations (5; 10; 15; 20; 25%) of Green Pomegranate Leaf Extract in Potato Dextrose Agar were evaluated for standardization of optimum concentration of leaf extract for getting maximum growth of *Cercospora punicae* in minimum possible time. The maximum radial growth of *C. punicae* (90 mm) was attained at 15 per cent Green Pomegranate Leaf Decoction Agar medium in 18 days and standardized as specific medium for conducting all the laboratory studies. The maximum growth was observed at temperature levels 25 ^{o}C (89.85 mm) which was significantly superior to all other temperatures levels *viz*., 15, 20, 30 and 35 ^{0}C on GPLDA medium. The pathogen grew best on pH 6 and the least growth occurred at pH 4.

Keywords: *Cercospora punicae*, effect, media, temperature, pH, *In-Vitro*

Introduction
Pomegranate is one of the main tropical and sub temperate fruit crops and is a good source of carbohydrates, and other minerals such as calcium, iron and sulphur. It is good source of Vitamin C and the predominant organic acid is citric acid. In India the total area under pomegranate cultivation was 234000 hectares with 2845'000 metric tonnes of cultivation (Anonymous, 2018) [2]. Due to its hardy nature, high yield with low maintenance costs and good quality maintenance, it cultivation has spread throughout the India (Khodade *et al*., 1990) [7]. Pomegranate is usually grown in dry and hot arid climate plants, but due to changing weather conditions and the expansion of its cultivation to wet areas, it has started to suffer from various diseases such as leaf spots, fruit rots, bacterial blight, anthracnose, etc. causing significant losses in the recent years (Anonymous, 1969) [1].
The leaf and fruit spots are incited by *Cercospora punicae*, *Xanthomonas axonopodis* pv. *punicae*, *Alternaria alternata* and *Colletotrichum gloeosporioides.* Similarly, fruit rots are caused by *Coniella granati*, *Phomopsis aucubicola* and *Phytophthora* sp. *Cercospora* leaf and fruit spot disease has emerged as a major problem in recent years in pomegranate cultivation in all important fruit growing districts of the Himachal Pradesh resulting in huge losses to the growers.
The *Cercospora* leaf and pomegranate spot or blotch was first described by Hennings in Japan in 1906 (Chupp, 1954) [4]. Symptoms tend to be circular to angular, dark reddish brown to almost black with diffused yellow halo on leaves and on fruits prominent dark brown, circular blotches which initially appear unequal (plate 1). In extreme infection, interfering with growth as a result of reduced production of photosynthates resulting in less production of fruit. Since pathogen growth on the PDA was very slow, none of the former workers recorded conidia development in the culture medium. Similarly, no one has identified the particular medium for the pathogen's rapid growth under exenic culture so far, and has identified only that yield PDA growth of about 2 cm under laboratory conditions (Wolf, 1927) [12]. Therefore the present study evaluates the effect of *invitro* media, pH and temperature on growth and sporulation of *Cercospora punicae*.

Plate 1: Symptoms of *C. Punicae* on fruit and leaves

Materials and methods

Isolation and identification of *Cercospora punicae*

The causal organism of *Cercospora* leaf and fruit spot was isolated from infected pomegranate leaves showing typical symptoms by following the tissue isolation technique on PDA and incubated at 25±1 ºC for 18 days. Identification was done as per morphological characters given in Illustrated Genera of Imperfect Fungi and as described by Wolf (1927) [12]. The identity of the culture was also got confirmed from NRC, Pomegranate Solapur, Maharashtra

Effect of various culture media, temperature and pH on growth of *Cercospora punicae*

Growth and sporulation of *Cercospora punicae* has been calculated on 5 strong media *viz*. Czapek Dox Agar (CDA), Green Pomegranate Leaf Decoction Agar (GPLDA), V8 Juice Agar (V8JA), and Oat Meal Agar (OMA). Green Pomegranate Leaf Decoction Agar (GPLDA) was developed by inserting various quantities of green pomegranate leaf extract into Dextrose Agar potato. Fresh leaves of pomegranate were gathered and washed first in the tap water, then in distilled water. 1000 g of fresh samples were chopped and then pounded with 1000 ml sterile distilled water (1: 1 w / v) and grinded. The extract was filtered through two layers of muslin cloth. Tyndallization sterilised the leaf extract to 100°C for 3 consecutive days. Various concentrations of pomegranate leaf extract (5, 10, 15, 20 and 25 per cent) were applied to Potato Dextrose Agar and poured into petriplates. The concentration at which best growth of *Cercospora punicae* observed was selected as the specific medium for best growth and conducting further experiments. Based on preliminary studies on best media the effect of temperature on growth and sporulation was investigated on GPLDA (Green Pomegranate Leaf Decoction Agar) incubated at 15, 20, 25, 30 and 35^{0}C. The effect of pH on growth and sporulation was also studied on GPLDA. The pH of the medium was adjusted to 4, 5, 6, 7, and 8 using 1M HCL and 1M NaOH prior to autoclaving. Mycelial discs (5 mm) were cut from 14 days old culture and a disc was aseptically mounted on each sterile Petri plate containing 20 ml of the culture medium. After 18 days of incubation, average colony diameter was observed, and the differences shown by treatments in different experiments were analyzed for their significance using standard statistical procedures as described by Gomez and Gomez (1984) [6].

Results and discussions

Fungus grew very slow on Potato Dextrose Agar where as it produced uniform dense colonies on Green Pomegranate Leaf Decoction Agar Medium. The surface of the colonies in contact with the medium was olivaceous in colour and the exposed surface was smoky (Plate 2). The mycelium was densely compacted except at the exposed surface. Fungus grew very slowly on Potato Dextrose Agar while on Green Pomegranate Leaf Decoction Agar Medium it formed uniform dense colonies. Except at the exposed surface the mycelium was tightly compacted. Branched hyphae, 2–3 µm wide, septate, constricted at septa, distance of 6–10 µm from septa. The fungal hypha was light brown in colour, septate and unbranched at 40X under compound microscope. Hyphae scale 2–9 µm thick, septate, septate constricted, gap between septa 5–26 µm, brownish or subhyaline, wall 0.3–1 µm thick and smooth. In culture Conidia was not formed.

The result on the effect of different types of media on of *C. punicae* presented in Table 1 show that there was significant difference (P=0.05) between growth on the four media investigated with the highest vegetative growth Since, the pathogen growth was very slow on the PDA, therefore different media were tried to find out the best medium for the quick growth. Out of five different media evaluated maximum radial growth of *C. punicae* was recorded on Green Pomegranate Leaf Decoction Agar media 5% (57.50 mm), followed by V8 Juice Agar (21.50 mm) and Oat Meal Agar (18.87 mm). However, the minimum radial growth was observed on Czapek Dox Agar (15.75 mm), and Potato Dextrose Agar (18.00 mm). Wolf (1927) [12] observed the growth of the *C. punicae* colonies on potato-dextrose agar, and about a month was required for the production of a colony of 20mm diameter. Five different concentrations (5; 10; 15; 20; 25%) of Green Pomegranate Leaf Extract in Potato Dextrose Agar were evaluated for standardization of optimum concentration of leaf extract for getting maximum growth of *Cercospora punicae* in minimum possible time (plate 2). The maximum radial growth of *C. punicae* (90 mm) was attained in 18 days at 15 per cent in Green Pomegranate Leaf Decoction Agar medium 15% and it was selected for conducting all the laboratory studies. Kilpatrick and Johnson (1956) [9] observed good growth and sporulation of *Cercospora* species on carrot leaf decoction agar. The different temperature levels *viz*., 15, 20, 25, 30 and 35 ^{0}C were also evaluated to know the suitable temperature for the maximum growth of the fungus, the maximum mean colony diameter (mm) of the fungus was observed at a levels 25 ºC (89.85 mm) which was significantly superior to all other temperatures on GPLDA medium. Results of present studies are in conformity with the findings of Phengsintham *et al*. (2011) [11] who also reported maximum growth of *C. punicae* at 25 °C. Similarly out of five pH levels evaluated maximum mean colony diameter (mm) of the fungus was obtained at pH 6 (90.00 mm) which was significantly higher than the growth at other pH levels followed by pH 5 (60.87 mm) and pH 7(38.87 mm). Dange and Patel (1968) [5] reported that *C. beticola* grew between 3.1 and 9.1, but the best growth occurred at pH 6. Present investigation indicated that, with the decreases or increases in pH level from the optimum, the rate of growth gradually decreased. Slow growth on PDA medium was time consuming to conduct any *invitro* investigation on

the fungus. Findings of this study will help researcher to grow the fungus very quick (90 mm) in 18 days. The rapid growth of *Cercospora punicae* on the GLPDA medium would save their time for *in vitro* experiments and help fill gaps in the literature that have not yet been adequately described previously.

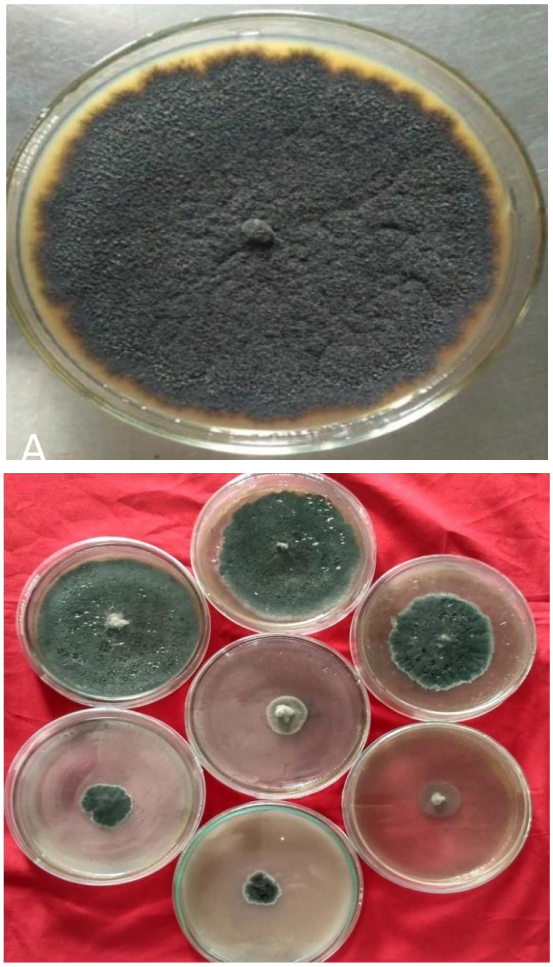

Plate 2: Culture of C. Punicae on GPLDA medium and its different mycelial growth on 15% GPLDA, 10% GPLDA, 5% GPLDA Czapek agar, oat meal PDA and V8 Liuice agar

Table 1: Response of different solid media for the growth of *C. punicae* under *in vitro* conditions

Media	Mean colony diameter (mm)
Green Pomegranate Leaf Decoction Agar (5%)	57.50
V8Juice Agar	21.50
Czapek's Agar	15.75
Potato Dextrose Agar	18.00
Oat Meal Agar	18.87
C.D.$_{0.05}$	1.56
SE±	0.51

Table 2: Effect of different concentrations of pomegranate leaf extract on the growth of *C. punicae* under *in vitro* conditions

Media	Mean colony diameter (mm)
Green Pomegranate Leaf Decoction Agar (5%)	57.50
Green Pomegranate Leaf Decoction Agar (10%)	80.50
Green Pomegranate Leaf Decoction Agar (15%)	90.00
Green Pomegranate Leaf Decoction Agar (20%)	90.00
Green Pomegranate Leaf Decoction Agar (25%)	90.00
C.D.$_{0.05}$	2.12
SE±	0.68

Table 3: Effect of temperature levels on growth of *C. punicae* under *in vitro* conditions

Temperature (°C)	Mean colony diameter (mm)
15	27.87
20	55.37
25	89.85
30	37.37
35	21.62
C.D.$_{0.05}$	1.73
SE±	0.56

Table 4: Effect of different pH levels on growth of *C. punicae* under *in vitro* conditions

pH level	Mean colony diameter (mm)
4	24.37
5	60.87
6	90.00
7	38.87
8	25.25
C.D.$_{0.05}$	1.16
SE±	0.38

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