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In vitro cell selection of chickpea against culture filtrate of *R. bataticola* with different concentrations

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Abstract
In a procedure of *in-vitro* callus multiplication for resistant callus to *Rhizoctonia bataticola*., it was observed that the fast growth of callus cultures were established only from seeds of chickpea. Which are treated with 0.2% bavistin for 6 min, 0.1% mercury chloride for 2 min and 20% sodium hypochlorite for 5min. Pale, friable white color growth of callus was obtained after 30-35 days on MS basal medium containing 2.0 mg/l NAA and 0.5 mg/l BAP and screened *in-vitro* for resistance to Millipore-filtered purified culture produced by the fungus *R. bataticola* for creating resistance to dry root rot disease in chickpea. Calli were challenged by different concentrations of culture filtrate of *R. bataticola*. A cell survival rate of 6.66% at a 18% selective dose of culture filtrate was achieved in unselected calli while at>18% concentration of culture filtrate, results in the death of 100% calli after 3 weeks. However, calli survived within a range of 30 to 65% when subjected to 10-2% fungal culture filtrate indicating low survival rate at higher concentration and high survival rate at lower concentration.

Keywords: fungus, MS medium, plant growth regulator, selective medium

Introduction

Chickpea, *Cicer arietinum* L. is one of the most important pulse crop of India. It is cultivated in about 8.56 million hectares with a production of 7.35 million tons and productivity 858 kg per hectare (Anonymous 2016). The average production of chickpea is 15-20 quintal per hectare which is low in spite of high yielding varieties and new agronomic practices. The reasons of low yield are so many, apart from other reasons the main cause of low yield of this crop is the incidence of diseases. In addition, legumes are the main, and at times, the only source of protein and essential amino acid for the inhabitants of developing countries where protein deficiency is common (Mayer, 1976).

In legumes, chick pea is one of the most important grain legumes used for human food and animal feed in developing countries and is a rich source of dietary protein (Singh, 1990). Chickpeas are a good source of zinc, phosphorus, iron and certain water soluble vitamins. They are also very high in dietary fiber and thus are a healthy food source of carbohydrates for persons with insulin sensitivity or diabetes (Hulse, 1991) [13].

Gram or Chickpea (*Cicer arietinum L.*), a member of family Fabaceae, is an ancient self pollinated leguminous crop, diploid annual (2N=16 chromosomes) grown since 7000BC, in different areas of the world. It is ranked 3rd after common bean (*Phaseolus vulgaris L.*) and pea (*Pisum sativum L.*) but its cultivation is mainly concentrated in semi-arid environments (Saxena, 1990).

Chickpea is an important source of protein. The protein content of chickpea is 22 %. It is usually supplemented with cereals to form balanced diet. Compared to the other sources of protein, pulses are the cheapest source and have been called “Poor man’s meat”. Chickpea proteins are rich in essential amino acid "Lysine" which is generally absent in food grains (Ahmed, 1984).

Among the diseases of chickpea, dry root rots emerging as the most destructive constraint to chickpea productivity and production, Asthe disease is more prevalent during hot temperature of 30 to 35°C and low soil moisture conditions. Dry root rot caused by *Rhizoctonia bataticola* (Taub.) Butler [Pycnidial stage: *Macrophomina phaseolina*(Tassi) Goid] is a soil and seed borne necrotrophic fungal pathogen that has a global distribution, which can infect more than 284 plant species throughout the world including monocot and dicots (Farr *et al*., 1995) [8].

Besides providing protein to the diet, legumes have served the purpose of adding valuable nitrogen and organic matter to the soil and provide rich fodders to the milk and draft animals. The low level of protein in the daily diets of the people of the developing countries is the major factor responsible for malnutrition than hunger.

India is the world leader in chickpea production followed by Pakistan. The chickpea crop is attacked by 172 pathogens (67 fungi, 22 viruses, 3 bacteria, 80 nematodes and mycoplasma) from all over the world. Among all, only a few of them have the potential to devastate the crops. Some of the serious disease in order of their importance are wilt (*Fusarium oxysporum f. sp. ciceri*) wet root rot (*Rhizoctonia solani*), dry root rot (*Rhizoctonia bataticola*) Ascochyta blight (*Ascochyta rabiei*) and collar rot (*Sclerotium rolfsi*) (Ravichandran *et al.*, 2014) [29].

The heavy demand created by the pressure of increasing population in the developing world requires a tremendous scientific effort to meet the requirements of food, fiber, fuel and other necessities of life. Since the conventional techniques employed in crop improvement may not keep pace with the demands of the increasing population and decreasing land resources, the importance of *in vitro*

technologies in crop improvement has great relevance. Recent advances made in the field of tissue culture have brought about new emerging technologies for crop improvement.

Micro propagation offers the potential to produce thousands or even millions of plants per annum and to produce disease free plants (Dry root rot of chickpea). Application of tissue culture techniques for genetic up gradation of economically important plants has been reported (Scowcraft and Ryan, 1985) [35].

More recently, plant biotechnology and molecular biology has emerged as spectacular discipline of life science. It has offered unprecedented opportunities and promises for the development of human resource and economic benefits (Sukapinda, 1993) [39].

In-vitro culture is a promising tool for selection of resistant mutants. Therefore the use of resistant cultivars may help to reduce the incidence of *R. bataticola*. In this paper, we describe a protocol for callus multiplication and to find the survival rate of callus against the pathogen *R.bataticola.*

Materials and methods

Establishment of callus cultures and multiplication

The seeds of the chickpea are selected for the development of callus against the pathogen *R.bataticola* Among all the explants used, the seeds were considered to provide the large amount of callus in a short period of time with the concentration of 2mg/l NAA+0.5 mg/l BAP

Leaves, Nodes, cotyledons, shoot tips were used as explants apart from the seeds for the establishment of callus cultures. The explants were first washed under the tap water to remove the dirt then rinse the explants in detergent solution for 20 min. Then wash under the running tap water for 1hr to remove all the traces of detergent. Now rinse the explant in distilled water for 5 to 6 times. The following process should be continued in the laminar air flow chamber. Surface sterilize the explants with 20% sodium hypochlorite for 5min and washed 3-4 times with sterilized distilled water so as to remove the soil contaminants. Thereafter these were subsequently treated with 0.1% mercuric chloride solution for different time intervals and washed 3-4 times with sterilized distilled water. The treated explants were inoculated and cultured on modified Murashige and Skoog (1962) medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) viz. a-naphthalene acetic acid (NAA),2,4-dichlorophenoxy acetic acid (2,4-D), benzyl amino purine (BAP) and kinetin (Kn) for the establishment of callus cultures. All the cultures were kept in a culture room at 26 ± 2°C with a 16-hr photoperiod.

Fig 1: Disease incidence of *Rhizoctonia bataticola* under field conditions

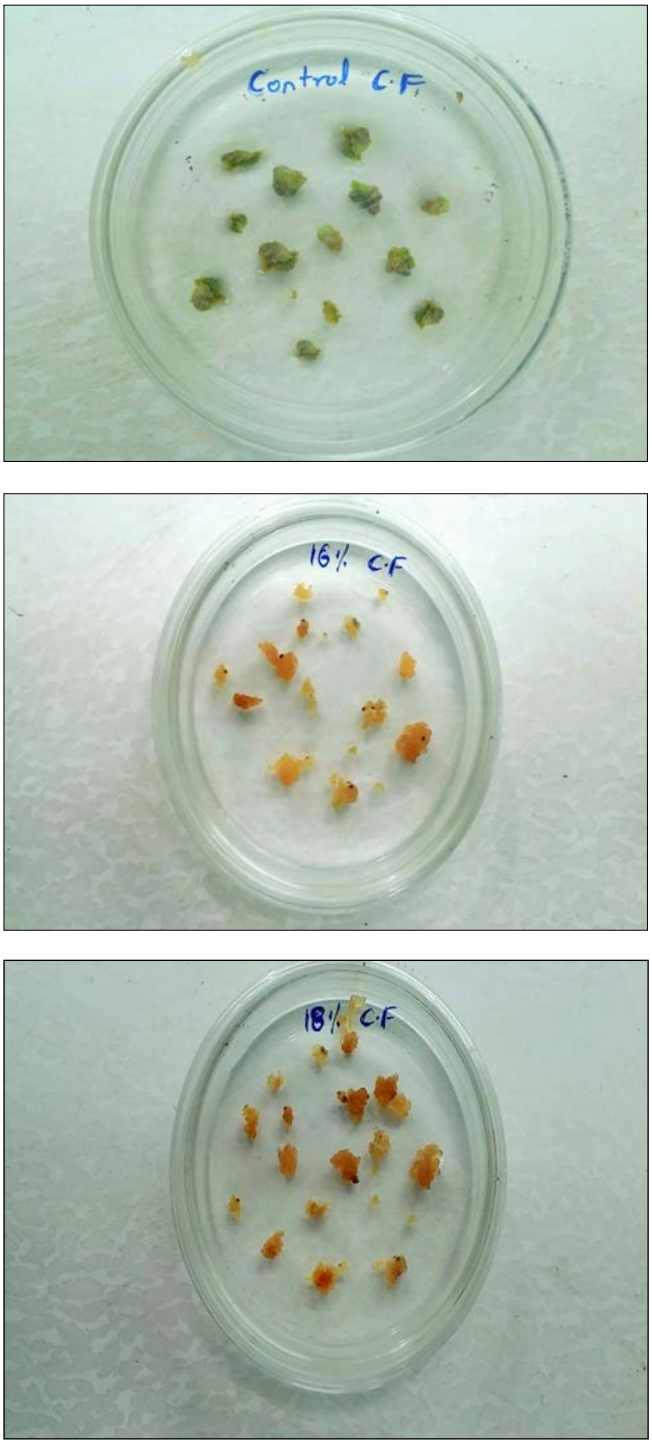

Fig 2: Callus multiplication of resistant calli of chickpea against *Rhizoctonia bataticola*

(A) Callus on normal MS + 2.0 mg/l NAA and 0.5 mg/l BAP
(B) Callus on selective medium with 16 % culture filtrate of *Rhizoctonia bataticola* (After 3 weeks)
(C) Callus on selective medium with 18% of culture filtrate of *Rhizoctonia bataticola* (After 3 weeks)

Preparation of culture filtrate

The fungus (*R. bataticola)* was isolated from infected roots of chickpea plant showing dry root rot symptoms under field conditions (Fig. 1). The pathogen was identified by the Department of Plant Pathology of the University of SHUATS. The diseased roots, after washing under running water, were cut into small pieces (0.3-0.5 cm) and treated with 0.1% mercuric chloride for about 2-3 min. These pieces, after washing with sterile distilled water (2-3 times), were aseptically placed on PDA (potato dextrose agar) medium prepared as per the constituents mentioned by Tuite 1969) and incubated at 25°C for one week. The isolated axenic culture of *R. bataticola* was maintained (at 25°C in incubator for 10-15 days) on PDA medium in test tubes (10-15 ml in borosil tubes) for uniform growth and used in different experiments. The pure culture of *R.bataticola* was cultured in liquid czapek's dox medium to obtain culture filtrate (Tuite 1969) [41]. The inoculated Erlenmeyer flasks (Fig.3) were kept in an orbital shaker incubator at 25°C and 100 rpm for about 30-35 days. The preparation of fungus culture filtrate was carried out in three distinct phases to remove mycelia (Nyange *et al*. 1997) [26]. The coarse filtration of fungal suspension was carried out through Whatman No. 42 filter paper following centrifugation at 10,000 rpm for 15 min and finally subjected to sterilization of culture filtrate through 0.22 µm. After filter sterilization, the culture filtrate was kept in the culture room for 48hrs and stored in a refrigerator at 4°C. The supernatant was used for screening the cells for resistance against fungal toxin using various dilutions for testing the toxicity of the culture filtrate, small pieces of callus were first macerated mechanically to separate single cells and then put in culture filtrate and kept for 48 hrs. Macerated callus in czapek's dox medium was kept as control. After 48 hrs both samples (treated and control) were stained with 0.1% erythrocin B (which stains only dead cells) and then seen under light micros- cope for confirming its viability which indicated the toxicity of culture filtrate.

Fig 3: Growth of *Rhizoctonia bataticola* in liquid czapek's dox medium.

***In-vitro* selection of resistant callus**

The actively growing callus were challenged to different concentrations of fungal culture filtrate by mixing the purified culture filtrate of fungus with sterilized molten MS medium to obtain several concentrations (v/v): 0, 2, 4, 6, 10, 16, 18, 20 %. About 20 ml of medium, after thoroughly mixing with culture filtrate, was poured in pre-sterilized Petri dishes under aseptic conditions. The MS medium used was supplemented with standardized concentrations of PGRs used for maintenance of the callus. Callus was cut into small pieces of about 20 mg each and then inoculated into selective media of different concentrations under a laminar flow chamber. The growth of the cells was monitored by their ability to divide and form colonies. The highest concentrations of culture filtrate at which calli

survived was recorded. The pale green colour of callus depicted the presence of living cells whereas dark brown color indicated dead cells (Fig. 2). The surviving calli were further sub-cultured on callus maintenance medium (MS basal +2.0 mg/l NAA and 0.5 mg/l BAP) then transferred to the same medium at 18% concentration of the selective dose of culture filtrate of the fungus. The resistance capacity of finally selected callus cultures was compared with previously non-selected callus cultures (control). Surviving callus cultures were separated for shoot formation only when same batch of culture filtrate caused greater than 88% survival rate under control to maintained the regeneration potential of calli.

Results and Discussion

Induction of callus and differentiation

For the establishment of callus cultures from explants, such as seeds, leaf tips, nodes, (0.5-0.7 cm in size) were treated with 0.2% bavistin for 6 min followed by treatment with 0.1% $HgCl_2$ for 2 min and 20%NaOCl for 5 min resulted in the survival of 88.33% uncontaminated cultures, whereas leaf explants exposedto10 min of bavistin (0.2%) and 4 min of $HgCl_2$ (0.1%) and 8 min of NaOCl (20%) died. Decreasing the time of treatment of either bavistin or sodium hypochlorite or Mercuric chloride resulted in less percentage of uncontaminated cultures while, longer duration of this treatment resulted in death of the explants. Although exposure of explants to 4 min in 0.2% bavisitin, 5 min in 20% NaOCl, and 2 min in 0.1% $HgCl_2$resulted in 81.66% of uncontaminated leaf explants. Callus formation was maximum on MS medium containing 2.0 mg/l NAA and 0.5 mg/l BAP.

About 10-14 days were required for callus initiation and 40-45 days for full growth of the callus. The callus formed from the cut ends of the initial explants was separated and sub- cultured onto fresh medium of the same composition i.e. MS basal medium+ 2.0 mg/l NAA and 0.5 mg/l BAP. This medium was subsequently used for the multiplication and maintenance of the callus culture (Fig. 2).

In-vitro selection of resistant callus

The incubated callus cultures along with fungal culture filtrate showed maximum (81.66%) calli survival at minimum (2%) concentration of culture filtrate. At 20% concentration of culture filtrate, 100% of the cells died in unselected calli whereas in selected cell cultures 6.66% survival of calli was observed at 18% concentration (Table 1). Callus that was tolerant to 18% of culture was further multiplied on callus initiation and maintenance medium. The culture was passed through three cycles of selection on this medium. In each selection cycle, the number of surviving calli decreased and finally selected calli grew better on medium with fungal toxin. The possibility of using either pathotoxin or partially purified culture filtrate of the pathogen as a screening agent for disease resistance *in-vitro* was first tested by Carlson (1973) [5] for wild fire of tobacco caused by *Pseudomonas syringae* pv. *tabaci*. This is also true for most of the *Fusarium* spp. filtrates used successfully in selection of alfalfa, tomato and carnation callus cultures (Hartman *et al.* 1984; Shahin and Spiney 1986; Arcioni *et al.*1987; Mosquera *et al.* 1999) [12, 36, 1, 24]. Various other researchers also reported cell selection based on toxins/culture filtrate of serious pathogens, e.g. *Fusarium oxsporum* f.sp. *dianthi* (pathotype II and IV) (Mercuri *et al.* 1992; Sanjuan *et al.* 2001) [33], *Alternaria dianthi* (Mehta *et al.* 2007) [21], *Helmin- thosporium sativum* (Ling *et al.* 1985; Rines and Luke 1985; Chawla and Wenzel 1987) [17, 30, 6], *Phoma lingam* (Sacristan 1982; Sjodin and Glimelius 1989), *Phytopthora infestans* (Behnke 1979, 1980; Rosati *et al.* 1990) [3, 4, 32], *Cercosporidium personatum* (Venkatchalam *et al.* 1998) in carnation, rice, oat, wheat, *Brassica* spp., potato and groundnut crops,etc.

Implicatons and Future Perspectives

From the present studies it can be concluded that culture filtrate of *Rhizoctonia bataticola* was injurious to the survival of callus of chickpea beyond 18 per cent. About 40 to 80 per cent survival rate of the callus was recorded when subjected to 2 to 10 per cent concentration of culture filtrate. However resistant plants could be obtained at 18% concentration of culture filtrate of the fungus for mass propagation and for use in breeding program seed was found to be the best explant for the establishment of callus cultures and maximum callus growth was obtained on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP.

Table 2: Effect of surface sterilants on percentage survival of ex-plantsof chickpea'after15days

Treatment	0.2% Bavistin	20% NaOCl	0.1% $Hgcl_2$	percent uncontaminated
T_1	2	5	2	48.33
T_2	4	5	2	81.66
T_3	6	5	2	88.33
T_4	8	6	3	36.66
T_5	10	8	2	0.00

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