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In vitro Selection for Wilt Resistance in Guava (*Psidium guajava* L.) cv. Allahabad Safeda

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Abstract: *In vitro* selection is a feasible method for developing wilt resistant or tolerant genotypes of guava. Unlike other crops, this approach has not been well established for guava. *In vitro* selection system against *Fusarium* wilt was employed with the aim to establish a recurrent selection system in guava (*Psidium guajava* L.) both at the cellular and plant level. *In vitro* somatic embryogenesis derived plantlets of guava cv. Allahabad Safeda were screened against *Fusarium* sp. culture filtrate at various concentrations of (0, 5, 25, 50 and 100%, v/v) supplemented in Murashige and Skoog (MS) basal liquid medium up to four selection cycles. The effect of *Fusarium* culture filtrate on plantlet survival and proliferation were observed to be directly related to the concentration in the selection media. The current study revealed that *in vitro* screening of guava plantlets using *Fusarium* sp. culture filtrate when exposed to the ten different culture filtrates at five concentrations of which; two of the culture filtrates of *Fusarium solani* (F2 and F15) shows maximum mortality of guava plantlets at 50% culture filtrate and later shows some level of tolerance to the culture filtrates. However, two of the culture filtrates F18 and F24 of *Fusarium oxysporum* f. sp. *psidii* shows maximum regeneration i.e., up to 100% even after fourth selection cycle. Current study revealed that *Fusarium* sp. culture filtrate can be potentially employed as a potent selection agent for carrying out *in vitro* selection approach against wilt disease of guava. The successful recurrent selection system developed *in vitro* on the basis of current research can probably help in ameliorate the problem of wilt in guava.

Key words: Disease resistance, culture filtrate, wilt, *in vitro* selection, plantlet

INTRODUCTION

Guava (*Psidium guajava* L.) belongs to the family Myrtaceae which has more than 80 genera and 3,000 species distributed throughout the tropics and subtropics (Chandra *et al*., 2010a). It is regarded as nutritionally valuable and remunerative crop used for both, table and processing purposes. It is liked by fruit growers due to its wide adaptability and higher return per unit area. But its successful cultivation is hampered by a number of biotic and abiotic factors. Among the biotic factors, diseases take a heavy toll. Production of guava has been severely affected worldwide by a soil borne disease called wilt. The disease was first reported in India by Das Gupta and Rai (1947). Wilt of guava is one of the most devastating problems faced by the orchardists in India, as the disease is totally uncontrolled through chemical or physical measures (Misra, 2006). Control measure other than eradication of diseased trees, were not fully successful. Although several fungal pathogens have been reported as the causal agents of wilt in guava by different workers but *Fusarium oxysporum* f. sp. *psidii* and *Fusarium solani* were found to be the most important pathogen associated with wilt (Misra, 2006; Misra and Gupta, 2007; Gupta *et al*., 2010). However, *Fusarium solani* is the most virulent and pathogenic fungi in nature most commonly found wherever the guava wilt disease is to be reported (Gupta *et al*., 2010). First external symptoms of disease are the appearance of yellow coloration with slight curling of leaves of the terminal branches. At later stage, plants show unthriftiness with yellow to reddish discolouration of the leaves and subsequently its premature shedding. Fruits of all the affected branches remain underdeveloped, hard and stony (Misra, 2006). Later on, the entire plant defoliates and dies. Mainly two types of wilt symptoms are identified-slow wilt and sudden wilt (Chakraborty and Singh, 1989). Substantial research work has been done on wilt disease of guava regarding chemical and biological controls through botanicals and biocontrol agents but, no workable solution has been

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successful yet. Wilt infected mother plants of guava are playing major role in spreading the disease beyond leaps and bounds. Therefore, it is of the utmost importance to develop wilt resistant guava cultivar in days to come otherwise the guava industry may collapse (Chandra and Mishra, 2007; Chandra *et al*., 2010a). Development of resistant varieties is thought to be the most viable strategy to overcome this problem. Selection for disease resistance breeding in fruit crops is an alternative to genetic engineering (Jayasankar and Litz, 1998; Hartman *et al*., 2004; Chandra and Mishra, 2007; Chandra *et al*., 2010b). This *in vitro* selection approach is easy to use and not encumbered by intellectual property issues and social concerns currently inhibiting development of transgenic crops. Thus, it is an attractive adjunct to the already existing crop improvement strategies through biotechnological interventions. The *in vitro* selection approach to select disease resistant/tolerant lines in culture has been to use phytotoxic fungal culture filtrate or purified toxins produced by the pathogen (Svabova and Lebeda, 2005; Sengar *et al*., 2009). Earlier studies on *in vitro* selection for disease resistance trait among the fruit crops viz., peach, lemon, grape, banana and mango were successfully carried out. These studies suggested the use of *in vitro* selection as potential approach for developing enhanced resistance to phytopathogens. The developmental mechanisms for obtaining disease resistance through *in vitro* selection remain obscure. In case of guava; for the first time a wilt resistant rootstock has been developed successfully using *in vitro* cellular selection system in South Africa against *Penicillium* sp. (Vos *et al*., 1998). In India, only a preliminary study of *in vitro* selection using fungal culture filtrates was initiated for the development of a wilt resistant rootstock for *Psidium* species (Bajpai *et al*., 2007) and so far no reports were available.

The current investigation aims with the objective to explore the effects of *Fusarium* sp. culture filtrate as a potent selection agent on *in vitro* regenerated guava plantlets to develop a recurrent selection system in guava which could be of great significance in further development of a wilt resistant genotype.

MATERIALS AND METHODS

The current research investigation was carried out at Biotechnology Laboratory, Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow (U.P.), India during January 2009 to March, 2011.

The immature fruits (70 days after post anthesis) of guava (*Psidium guajava* L.) were collected as explant source from guava orchard at C.I.S.H., Rehmankhera, Lucknow, India. The guava cv. used in the present study was Allahabad Safeda. Seventy days old immature guava fruits were brought to the laboratory and surface sterilized with water and then soaked in $HgCl_2$ (0.1%) for 3 min followed by thorough washing with distilled water in order to remove traces of mercuric chloride. Under aseptic conditions flame sterilized the fruit using 90% alcohol and then cut the fruit into two halves and scoops out the immature zygotic embryos (Akhtar, 2010) embedded in the mesocarp of the fruit and inoculate into the basal medium.

Culture initiation: The immature zygotic embryos, 70 days or 10 weeks after post anthesis were used as explant material for inducing somatic embryogenesis in guava. The zygotic embryos were inoculated in induction medium containing (Murashige and Skoog, 1962) medium supplemented with 2.0 mg L^{-1} 2, 4-D, 400 mg L^{-1} L-glutamine, 60 g L^{-1} sucrose and 0.8% (w/v) agar-agar. The medium prepared adjust pH 5.8 prior to autoclaving at 121°C at 15 lbs pressure for 20 min. The cultures were incubated under dark for inducing somatic embryogenesis and thereafter shifted the embryos to the regeneration media (0.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA) in the light for further regeneration and plantlet development. The plantlets developed were kept under artificial lightening at 24±2°C for 16/8 h dark. These somatic embryogenesis derived plantlets were now used as explant source for carrying out *in vitro* selection.

Pathogen culture: The pathogenic *Fusarium* sp. isolates were isolated from the rhizospheric regions of the wilt infected guava plant roots and grown on potato dextrose agar (PDA, Hi-Media) and incubated at 28±2°C for 7 days and were routinely transferred to medium at every 30 days or 4 weeks. The morphological and cultural characterizations of the pathogenic *Fusarium* sp. culture were compared with those mentioned by Booth (1971). The details of pathogenic pure cultures of *Fusarium solani* and *Fusarium oxysporum* f. sp. *psidii* were represented (Table 1). The pathogenic reference pure *Fusarium* sp. cultures were confirmed from Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, I.A.R.I., New Delhi for identification of *Fusarium solani* [ITCC No. 5208 (F20) and 5212 (F15)] and Microbial Type Culture Collection and GenBank (Chandigarh, Punjab) *Fusarium oxysporum* f. sp. *psidii* [MTCC No. 3326 (F24) and 3327 (F30)]. However, *Fusarium solani* F2, F12 and F20 (Booth, 1971) and *Fusarium oxysporum* f. sp. *psidii* F10, F18 and F38 (Booth, 1971).

Table 1: *Fusarium* sp. isolates used for *in vitro* selection studies against wilt of guava

Isolate No.	Location	Wilt type/ Pathogenicity (%)	Identification	Metabolite (colour) in culture
Fusarium oxysporum* f. sp. *psidii				
F10	Chandigarh	50	Booth (1971)	Light brown
F18	Ranchi	100	Booth (1971)	Brown
F24	Kanpur	50	MTCC 3326	Pale yellow
F30	Unnao	75	MTCC 3327	Pale yellow
F38	Rewa	50	Booth (1971)	Dark yellow
Fusarium solani				
F2	West Bengal	100	Booth (1971)	Violet
F12	Chandigarh	50	Booth (1971)	Pinkish
F15	Ajmer	100	ITCC 5212	Pinkish
F20	Puskar	100	ITCC 5208	Pinkish
F29	Unnao	100	Booth (1971)	Pink yellow

Preparation of *Fusarium* sp. culture filtrates: Potato Dextrose Agar (PDA) medium petri dishes were inoculated with *Fusarium* sp. isolates (Table 1) and incubate under dark at 28°C in BOD incubator. After one week 2 discs of 5 mm diameter (using cork borer) of pathogenic sporulated *Fusarium* sp. cultures were inoculated into the flasks containing potato dextrose broth under aseptic conditions. Incubate the inoculated broth culture at 28°C in orbital shaker at 120 rpm for 3 weeks or 21 days. Later, the cultures were taken out containing *Fusarium* sp. culture filtrate is now filtered through four layers of what man No.1 paper and then centrifuged for 10 min at 13000 rpm to remove the traces of mycelium and conidia. After this, the culture filtrate is again filtered through 0.22 μ millipore syringe filter and maintained pH 5.8 before use kept at -20°C before performing *in vitro* studies under aseptic conditions.

***In vitro* testing of pathogenicity:** *In vitro* regenerated guava leaves were used to determine the phytotoxic effect of *Fusarium* sp. cell free culture-filtrate. Leaf segments (approximately 20 mm) of 30 day-old *in vitro* regenerated guava plants were taken and punctured with a fine needle and 5 μL concentrated *Fusarium* sp. phytotoxic culture filtrate (90% v/v) was dropped onto the wounded area. All leaf segments were incubated on moist what man filter paper No. 1 (analytical grade) in sterilized petri dishes under continuous fluorescent light at 26±2°C. Sterile water and Potato dextrose broth were used as controls and each test was repeated three times. Symptoms of leaves were noted after 72 h of incubation.

Callus growth bioassay: Callus cultures of guava were established using immature zygotic embryos as initial explant for callusing from cultivar Allahabad Safeda and placed on Murashige and Skoog medium (Murashige and Skoog, 1962) basal medium supplemented with 2.0 mg L^{-1} 2, 4-D, 400 mg L^{-1}, 60 g L^{-1} sucrose and solidified using 8 g L^{-1} agar. Ten pieces of guava calli approx. Ten milligram were transferred to induction medium. The semisolid medium was supplemented with various concentrations of culture filtrate (0, 5, 25, 50 and 100%) and control without culture filtrate containing medium. The experiment consisted of three replicates for each experiment in a completely randomized design. Calluses were incubated for up to four week and symptoms were noticed and ranked into five categories: 1 = browning, 2 = friable callus, 3 = dried callus, 4 = deeply brown with no regeneration, 5 = compact and regenerating.

Culture procedure: *In vitro* somatic embryogenesis derived plantlets of guava cv. Allahabad Safeda were inoculated on full strength Murashige and Skoog (Hi-media) liquid media (devoid of any growth hormone) containing various concentrations of culture filtrate (0, 5, 25, 50 and 100%, v/v) of *Fusarium* sp. and control without culture filtrate. Ten selected pure pathogenic cultures of *Fusarium oxysporum* f. sp. *psidii* and *Fusarium solani* were used as selection agent for screening guava plantlets for developing wilt resistance under *in vitro* conditions. Each experiment was conducted in a completely randomized design with five replications and each experiment carried 50 explants/treatment and repeated at least thrice for further statistical analysis (O.P. Sheoron-A software).

***In vitro* selection using callus culture and plantlets:** Approximately 15-20 callus/treatment and at least 50 plantlets per treatment were taken and subjected to recurrent selection in Murashige and Skoog liquid media (without agar) culture with a modified culture medium containing 0, 25, 50 and 100% (v/v) fungal (*Fusarium* sp.) filtrate/culture filtrate. The MS liquid medium were prepared autoclaved and cooled at room temperature and the *Fusarium* sp. culture filtrate were amended into the liquid media after cold-filter-sterilization through 0.22 μ Millipore syringe filter and the pH of the culture filtrate was adjust to 5.8±2 before adding to the sterile MS liquid medium in order to eliminate loss of activity of culture filtrate during autoclaving. The callus cultures and plantlets were subculture once at every 10th day and during each subculture, fresh medium with culture filtrate/culture filtrate were used. After four and five such transfers, surviving callus or plantlets were recovered and cultured in medium without culture filtrate for further proliferation and those survived were considered as putative resistant and subjected to subsequent selection cycles. The cultures were incubated at 28±2°C in the dark for four selection cycles (each selection cycle of 21 days).

RESULTS

In the current study, ten most virulent/pathogenic cultures filtrates of *Fusarium* sp. five of *Fusarium oxysporum* f. sp. *psidii* and five of *Fusarium solani* selected for carrying out *in vitro* selection process. Of these ten culture filtrates; three of the culture filtrates (F2, F15 and F20) of *Fusarium solani* were proved to be a potent selection agent (Fig. 1, 2) shows promising selections in both the explants viz., callus and regenerated plantlets. The percentage survival of callus cultured on media containing culture filtrate at various concentrations decreases with increasing concentration, reaching a 0% survival at 100% culture filtrate (Fig. 3).

In some *Fusarium* sp. culture filtrate F18 and F24 (*Fusarium oxysporum* f. sp. *psidii*) a 100% plantlet regeneration or survival were noticed with increasing concentration of culture filtrate even after four selection cycles (Fig. 4d). Although, the shoot regeneration in treated plantlets was observed after 2 weeks of treatment. However, in case of explant callus regeneration frequency after two selection cycles were found varied significantly with respect to the concentration of *Fusarium* sp. culture filtrate. Lowest regeneration efficiency of callus was observed in 100% culture filtrate of *Fusarium* sp. as compared to the control. The gradual selection of the plantlets for resistance to higher concentration of culture filtrate were apparent as the plantlets finally surviving on the culture filtrate at higher concentration 50% of culture filtrate describes promising selections as well as shows maximum wilting symptoms (Fig. 4a-d) with higher concentrations resulted in a small proportion of viable shoots which may be adopted to develop resistant genotypes.

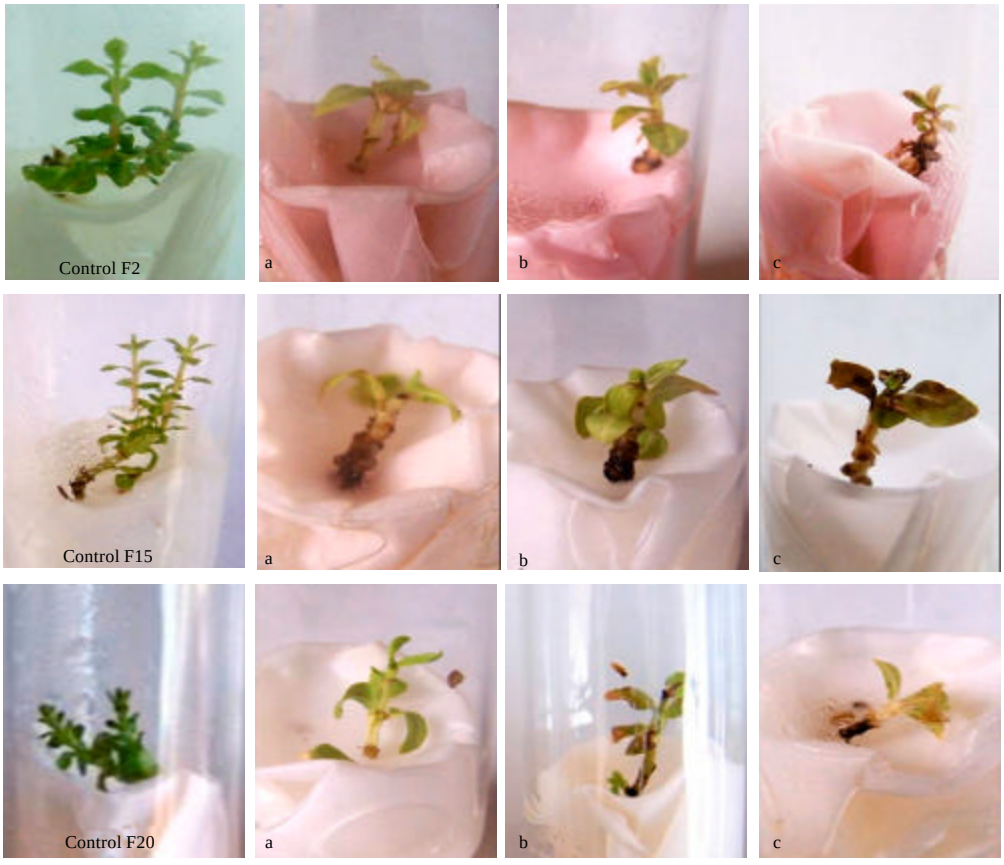

Fig. 1: *In vitro* selection in guava using culture filtrate of *Fusarium solani* (F2, F15 and F20) shows a series of wilting symptoms; F2a: Mottling of leaves and root zone, F2b: Browning of root zone, F2c: Drying of leaf at tip portion and ultimately death of plantlets, F15a: Mottling of root-zone, F15b: Browning and blackening of root-zone, F15c: Drying of upper leaves, F20a: Falling leaves, F20b: Leaves drying and falling of leaves and F20c: Drying of root zone and yellowing of leaves

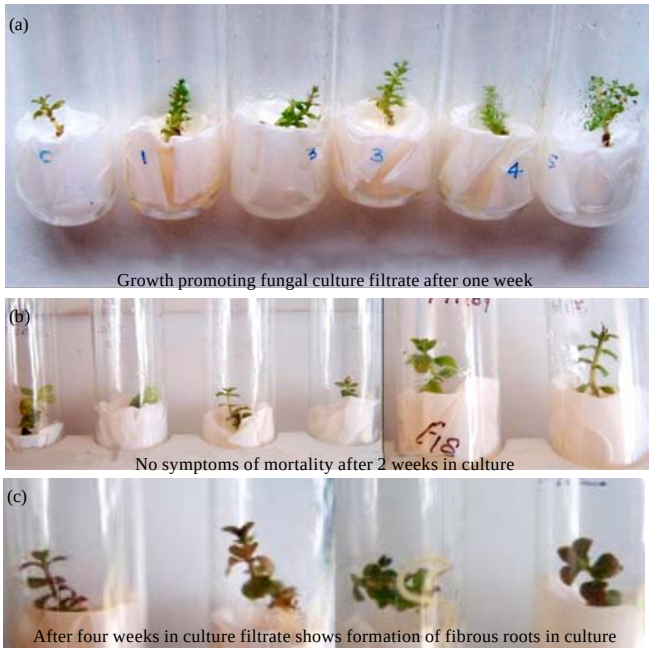

Fig. 2(a-c): Depicting *in vitro* selection system in guava using *Fusarium oxysporum* f. spp. *psidii* at 50% culture filtrate (F18) shows plant growth promoting activity and no wilting symptoms (Fig. 2a-c) formation of fibrous roots and growth of leaves

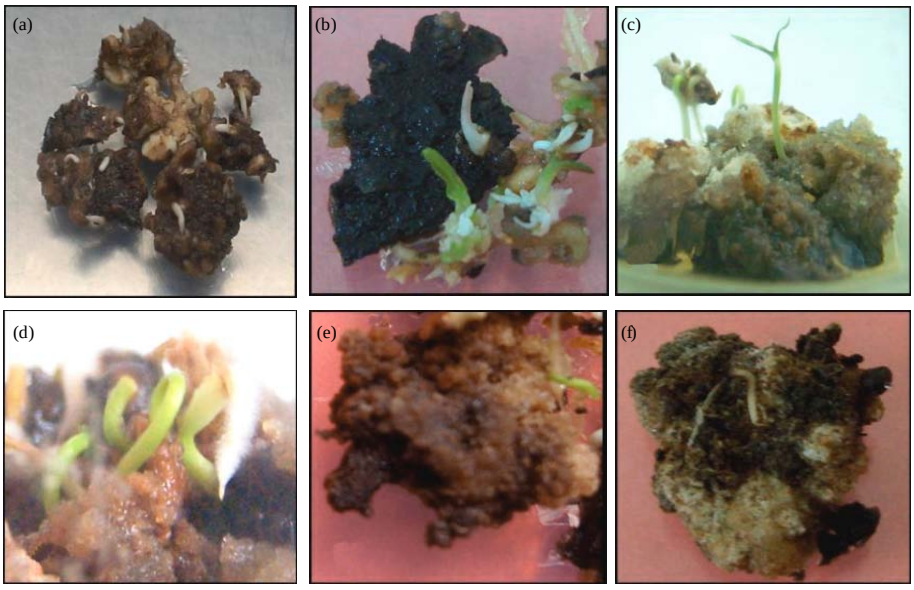

Fig. 3(a-f): Different stages of callus mortality symptoms when exposed to 50% *Fusarium solani* (F2) culture filtrate amended in medium shows maximum wilting, (a) browning of callus with loss of embryogenesis, (b) callus blackening, (c) friable and watery callus, (d, e) dying of fragile callus and (f) drying and death of callus tissue with no further regeneration

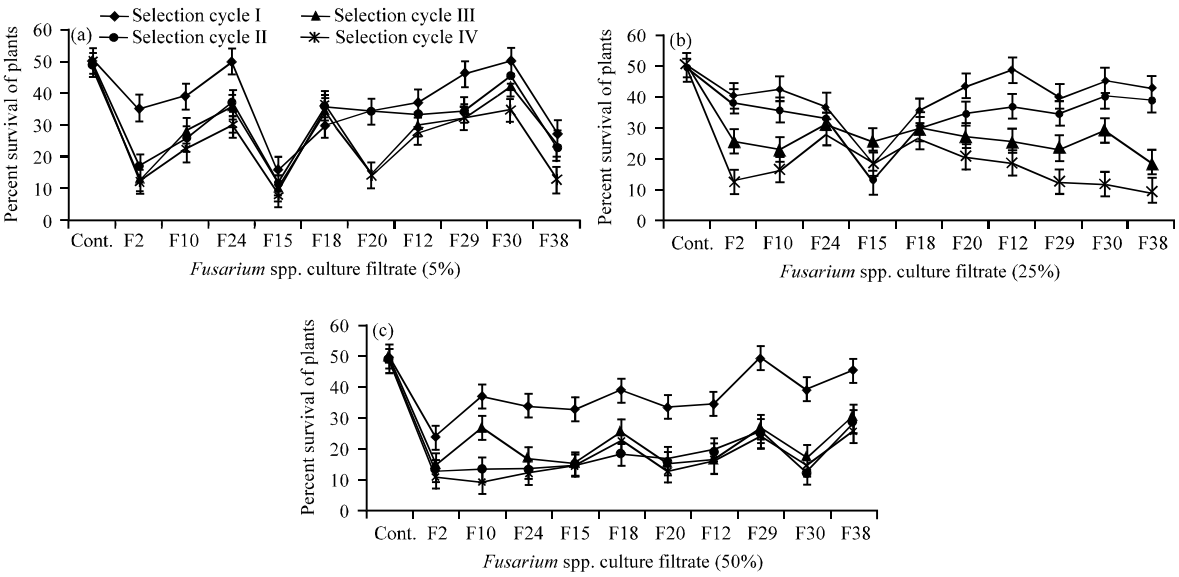

Fig. 4(a-c): *In vitro* screening of guava plantlets cv. Allahabad Safeda (*Psidium guajava* L.) using culture filtrates of 10 isolates of *Fusarium* spp., (a) Percent survival of plantlets at 5% culture filtrate, (b) Percent survival of plantlets at 25% culture filtrate and (c) Percent survival of plantlets at 50% culture filtrate during four selection cycles

According to the present findings *Fusarium solani* culture filtrate F2, F15 and F20 culture filtrate (Fig. 1) shows maximum wilting symptoms with increasing concentration starting from 0, 25, 50 and 100% conversely, in the controls. At control 100% plantlet regeneration was recorded and no symptoms of mortality (Fig. 4a-d). The plantlets selection were therefore carried out at the 50% culture filtrate level to exert maximum selection pressure that can allowed the recovery of resistant plantlets. Additionally, in the present study the other two culture filtrates of *Fusarium oxysporum* f. sp. *psidii* (F18 and F24) poor symptoms of wilting even after 30 days or fourth week of a selection cycle (Fig. 2). However, these two selections (F18 and F24) showed good regeneration at maximum (100%) concentration of culture filtrate after four weeks of selection (Fig. 4d). This assumes that *Fusarium oxysporum* f. sp. *psidii* isolates used in this study was non-pathogenic or avirulent as compared to the *Fusarium solani* which is registered as a most virulent pathogen causes serious damage to guava plants which is observed from this study. Pathologically, it has been proved that *Fusarium solani* is the most virulent and pathogenic in nature found commonly wherever wilt disease is to be reported (Gupta *et al*., 2010). The results of current study revealed that some 15-20% of guava calli shows ceased growth or become friable and become severely blackened and 80% calli was mortalized after first selection cycle (data not given). Figure 3a-f clearly demonstrates the callus mortality symptoms as well and the remaining or survived cultures exposed to selection pressure after first selection cycle shows poor regeneration or friability.

DISCUSSION

This study reports the first attempt to assess the potential of *Fusarium* sp. culture filtrate as a potent selection agent for establishing *in vitro* selection system in guava (*Psidium guajava* L.) cultivar Allahabad Safeda for resistance to *Fusarium* wilt. The data demonstrate that this approach could be potentially employed for screening and establishing large populations of the guava cultivars for resistance to *Fusarium* wilt in Indian sub-continent. Histopathological studies confined that no traces of *Fusarium* mycelium was found from the wilted guava plant. Presumably, it was concluded that the toxins released by the *Fusarium* sp. in the xylem vessels causes yellowing of upper leaves, wilting of stems and eventually resulting in the death of a plant (Beckman and Roberts, 1995). *In vitro* selection approach using *Fusarium* sp. culture filtrate appears as a promising tool to rectify specific defects to improve a desirable trait of highly adapted cultivars without involving a sexual cycle. Chandra *et al*. (2010a) reviewed the *in vitro* selection as candidate approach to select disease resistant lines in culture using phytotoxic fungal culture filtrate or purified

toxins produced by the pathogen (Behnke, 1979; Wenzel, 1985; Daub, 1986; Chawla and Wenzel, 1987; Jayasankar and Gray, 2003). The availability of a defined culture filtrate or pathogen culture filtrate has been advantageous for inculcating *in vitro* selection protocol. Culture filtrates that are known to play a role in pathogenesis are called pathotoxins (Bulk, 1991; Svabova and Lebeda, 2005). However, to be useful, toxins must be implicated in the disease development process which act at the cellular level and have a mode of action that allows recovery of resistance (Yoder, 1980; Daub, 1986; Hammerschlag, 1984; Svabova and Lebeda, 2005).

The correlation between guava genotype susceptibility and the toxicity of culture filtrates suggests that filtrates could be used as potent selection agent to screen *in vitro* regenerants for disease resistance. Tissue culture systems can provide a means of rapid screening for disease resistance when a culture filtrate is involved in disease development (Daub, 1986; Hammerschlag, 1984; El-Hadrami *et al*., 2005). However, it has been concluded from the present findings that *Fusarium* sp. culture filtrate can have enough potential to be used as a selection agent against wilt resistance in guava cv. Allahabad safeda. Accordingly, Jin *et al.* (1996) obtained culture filtrate from *Fusarium solani* which has pathogenic characteristic, inhibit the growth of soybean cultures which was susceptible to Sudden Death Syndrome (SDS) disease. Similarly, in case of apple (Raman and Goodwin, 2000), mango (Jayasankar *et al*., 1999), peach (Hammerschlag, 1988), strawberry (Toyoda *et al*., 1991), lemon (Gentile *et al*., 1992) and pineapple (Borras *et al*., 2001), banana (Matsumoto *et al*., 1999; Okole, 1995), grapevine (Jayasankar *et al*., 2000) *in vitro* selection system has been carried out successfully to develop a resistant lines and varieties. An important biotechnological aspect of crop improvement via an *in vitro* selection approach is that traits selected at the cellular and plant level must be expressed in the regenerated plants. Our results showed that guava plantlets selected using *Fusarium* sp. culture filtrate against *Fusarium* wilt at various concentrations on callus and *in vitro* regenerated plants exhibited improved resistance. It was also concluded from previous studies that A number of previous studies that toxins present in the culture filtrate were able to inhibit growth and survival of the plantlets of the host species were more sensitive to the toxin than those of non-host plants (Huang and Hartman, 1998). According to our findings, about 15% of the plantlets exhibited resistance to *Fusarium solani* and the plantlets having resistance were further shifted to green house conditions for hardening to be developed as resistant/tolerant plantlets. In both the susceptible and resistant plantlets, increasing the concentrations of culture filtrate increased the intensity of response which was measured by symptoms of wilt i.e., burning of upper leaves, leaf fall, mottling of leaf, browning of root-zone.

Hence, the present investigation illustrates the development of a recurrent selection system *in vitro* which can possibly an alternative solve towards the long withstanding problems in guava like susceptibility to *Fusarium* wilt of guava and which can be successfully addressed especially in popular and commercial variety like Allahabad Safeda which has desirable traits in all other aspects except resistance to *Fusarium* wilt disease. A stable *in vitro* selection system developed on the basis of the current findings can go a long way in eliminating single trait problem while retaining others and also, shortening the time to release a variety which otherwise would entail a long period of 10-12 years by conventional means.

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