Selection of leaf blight-resistant *Pelargonium graveolens* plants regenerated from callus resistant to a culture filtrate of *Alternaria alternata*

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Abstract

A protocol has been established for the induction of disease resistance in callus cultures of rose-scented geranium, *Pelargonium graveolens* cv. Hemanti against leaf blight disease caused by the fungal pathogen *Alternaria alternata*. The callus cultures were raised and maintained on semi-solid Murashige and Skoog’s (MS) medium supplemented with 10 mg l\(^{-1}\) Kn and 1.0 mg l\(^{-1}\) NAA. The calli were subjected to various concentrations of culture filtrate (0\%, 4\%, 8\%, 12\%, 16\% and 20\%) obtained from *A. alternata*. Resistant calli were selected and placed on regeneration medium (MS supplemented with 0.5 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) NAA). The regenerants were confirmed for *A. alternata* resistance by exposing their leaves to the same concentrations of culture filtrate as used previously. While the parental wild type demonstrated typical susceptibility, the leaves of putative resistant clones remained green and viable in the presence of toxin and regenerated shoots directly on the toxin-free regeneration medium (MS supplemented with 5 mg l\(^{-1}\) Kn and l mg l\(^{-1}\) NAA).

The above experiment demonstrated the induction of disease resistance in rose-scented geranium plants at the cellular level. This approach could be successfully exploited in raising new disease-resistant cultivars in geranium against various fungal pathogens.

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1. Introduction

The crop *Pelargonium graveolens*, also known as rose-scented geranium yields essential oil, extensively used in the cosmetic and perfume industry (Kumar et al., 1985; Bijalwan and Kediyal, 2006; Saxena et al., 2007). Approximately 700 tonne of geranium oil is produced annually worldwide (approximate yield of 40 kg ha\(^{-1}\)) (Khanuja et al., 2005; Bijalwan and Kediyal, 2006). Of the 100–120 tonne of geranium oil annually consumed in India, only about 5 tonne are produced locally (Bijalwan and Kediyal, 2006). Geranium oil production needs to be increased because geranium oil uses are expanding owing to its aromatic property as well as its recently discovered biological activities (Jalali-Heravi et al., 2006).

The geranium crop is generally cultivated in temperate areas of the world (Charlwood and Charlwood, 1991) including those in India, in the southern hilly tracts. However, looking at the increase in demand, it was realized that the area of cultivation of the geranium crop could be extended to the northern Indo Gangetic plains (Saxena et al., 2000). The crop is perennial in temperate areas but it must be grown annually in the plains because the plant cannot withstand heavy rains and high humidity. Additionally, high humidity encourages a number of fungal pathogens.
The geranium crop in India has been observed to be generally affected by four fungal pathogens. *Rhizoctonia solani* and *Fusarium oxysporum* var. *redolens* are highly prevalent in the southern hilly tracts, while *Alternaria alternata*, *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* cause severe leaf blight in the northern Indian plains (Alam et al., 2007).

*Alternaria* blight disease symptoms in geranium are characterized by the appearance of brown necrotic spots on the leaf margin. The necrosis spreads towards the midrib; as a result the leaf curls up and dries, affecting the herb yield. The disease reduces the essential oil yield by disrupting the foliar oil glands (Shukla et al., 2000). Though the spread of disease can be prevented to an extent with the use of copper fungicides, a long-term solution to this problem is the development of resistant cultivars in this crop. Because the crop is polyploid and highly sterile, traditional breeding cannot be carried out. Somatic mutation and genetic engineering techniques appear to be most suitable for incorporating disease resistance in such vegetatively propagated plants (Van den Bulk, 1991; Bi et al., 1999).

Somaclonal breeding programmes have proved extremely successful in developing disease-resistant cultivars in a number of crop species (Behnke, 1980a; Thanutong et al., 1983; Kintzios et al., 1996; Thakur et al., 2002). It has either been attempted at the plant level where a large population of plants, raised through *in vitro* callus cultures, have been screened for resistance in the field directly or via a more targeted approach of regeneration of disease-resistant plants through resistant callus cultures selected against fungal toxin (Heinz, 1973; Carlson, 1973; Behnke, 1980b; Rines, 1986; Hammerschlag, 1992).

The present paper reports experiments in which *P. graveolens* cv. Hemanti (Rao et al., 1999) somaclones, resistant to leaf blight caused by a fungal pathogen *A. alternata* have been isolated by regenerating calli from stem nodes, selected against the fungal toxin followed by early assessment of resistant phenotype among the regenerating shoots, using a leaf phytotoxicity bioassay.

2. Materials and methods

2.1. Callus production

Somaclones were induced from the Hemanti cultivar of rose-scented geranium, which was well adapted in the plains and was being commercially exploited. The stem explants were surface sterilized with 0.1% HgCl₂ for 3.5 min, thoroughly rinsed with sterilized distilled water and inoculated on semi-solid MS (Murashige and Skoog, 1962) medium supplemented with 10 mg l⁻¹ kinetin (Kn) and 1.0 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (NAA) in 250 ml broad-necked conical flasks (Saxena et al., 2000). The cultures were maintained under *in vitro* conditions at a temperature of 25±2°C under a 16 h light regime (80 μmol photon m⁻² s⁻¹) and 70% RH. The callus obtained after 6 weeks of culture was subcultured onto the above medium at 4-week intervals.

2.2. Maintenance and multiplication of the pathogen

*A. alternata* (isolate no. 3448.97, Indian type culture collection IARI, New Delhi) was obtained from *P. graveolens* cv. Hemanti grown at CIMAP Farm, Lucknow, India. The isolate was multiplied at 23±2°C on PDA (200 g l⁻¹ potato, 20 g l⁻¹ dextrose and 20 g l⁻¹ agar) and maintained at 4°C on the same medium. The pH of the PDA medium was adjusted to 6.0. After inoculation, the cultures were incubated at 23±2°C in the dark for 7–10 d until a uniform mycelial growth was obtained.

2.3. Preparation of culture filtrate

The culture filtrate (CF) was prepared by inoculating 5 mm² pieces of the fungal mycelium in 11 Roux bottles containing 100 ml of liquid V₈ juice broth medium (Hi-Media Ltd., Mumbai, India) and incubated at 23±2°C for 25 d in the dark. After the incubation period, the mycelium obtained was filtered through four layers of cheese cloth and Whatman filter paper No. 1 and finally through a membrane filter with a pore size of 0.45 μm (Schleicher and Schull, West Germany). The pure CF was transparent yellow in appearance and maintained in a refrigerator at 4°C in dark. Thereupon, the 1000 ml culture filtrate was concentrated to 200 ml volume in a rotary flask evaporator under vacuum at 45°C. After concentration, the dark yellow CF (200 ml) was used to prepare a range of concentrations of toxic media (0%, 4%, 8%, 12%, 16% and 20% [v/v]) in liquid MS medium.

2.4. Testing the toxicity of the culture filtrate

Phytotoxicity of the culture filtrate was tested on root growth of germinating wheat seedlings (24 h old) obtained from *Triticum aestivum* var. K 65 and excised leaves of *P. graveolens* cv. Hemanti. Phytotoxic activity of CF was determined by the root growth inhibition bioassay as described by Wheeler et al. (1971) and modified by Shukla and Hussain (1987). Petri dishes (50 × 17 mm) containing 5 ml of diluted CF with five uniformly germinated wheat seedlings were incubated at 23±2°C for 48 h and growth of the primary roots was measured. The leaf lesion bioassay was carried out on leaves from 8-week-old plants of geranium. They were inoculated with diluted CF as 1:2, 1:4, 1:8 and 1:10 with water. An area of ca. 2 mm² on the upper leaf surface was gently scratched with a needle and 20 μl of toxin solution was applied. Three leaves from each plant were used for inoculation and each leaf was inoculated at four points. The corresponding un-inoculated medium was used as the control. Phytotoxic activity was based on the maximum dilution factor of the toxin required to cause chlorosis and yellowing on the host leaves after 48 h of incubation (Shukla et al., 1989).
2.5. Sensitivity and selection of calli for culture filtrate tolerance

The calli pieces (15 x 1 mm²) were inoculated into 100 ml conical flasks containing the toxic medium. Similar calli pieces inoculated into a toxin-free liquid MS medium served as control. The flasks were shaken in the dark at 80 rpm on a rotary shaker for a period of 7 d at 22 °C and observations were recorded every 24 h. The experiment had five replicates. The culture filtrate-treated calli were then placed on a semi-solid toxin-free MS medium containing 0.5 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA in order to obtain shoots. The regenerated shoots were excised after 6 weeks of culture and transferred to half-strength semi-solid MS medium containing 1 mg l⁻¹ of IBA for root induction. The rooted plants were maintained by regular subculture at an interval of 10–12 weeks. All the cultures were maintained at 25 ± 2 °C under a 16 h light regime (80 μmol photon m⁻² s⁻¹) and 70% RH. The regenerated shoots were observed for the following parameters: number and length of shoots, number of leaves borne on the plantlet and size of root system formed. None of the calli survived in the media containing toxin irrespective of the concentration beyond an exposure period of 7 d and did not even revive on subsequent transfer to a non-toxic medium. Therefore, the optimal time period for toxin treatment was kept constant at 7 d during further experimentation.

2.6. Phytotoxicity test

The excised leaves borne on the regenerants from the toxin-treated and untreated calli were tested for resistance/phytotoxicity to the culture filtrate of A. alternata by plating them on semi-solid MS media containing same concentration of CF as used for selecting resistant calli (4%, 8%, 12%, 16% and 20% [v/v]). The leaves that survived the media containing toxin irrespective of the concentration beyond an exposure period of 7 d and did not even revive on subsequent transfer to a non-toxic medium. Therefore, the optimal time period for toxin treatment was kept constant at 7 d during further experimentation.

2.7. In vivo testing of regenerated plants against the pathogen

The cuttings of the plants regenerated from the resistant callus and those from untreated control plants were screened for leaf blight resistance. The spore suspension was obtained from the 10-d-old colonies of A. alternata on PDA medium by scraping the conidia into a 0.1 M MgSO₄ solution. The spore concentration was adjusted at 1 x 10⁷ spores ml⁻¹ and Tween 20 (0.1%) was used as the sticking agent. The spore suspension was sprayed on three replicates of the leaves of cuttings. The inoculated cuttings were maintained under glasshouse conditions before as well as after inoculation at 25 ± 5 °C with 70% RH. They were observed daily for 15 d following inoculation, and the disease symptoms were recorded. Resistance was classified in three categories, viz. I—resistant (0–2 min superficial lesions/leaf); II—moderately resistant (3–6 deep lesions/leaf); and III—susceptible (>6 deep lesions/leaf) according to Verma and Saharan (1994).

2.8. Statistical analysis of the experimental data

The observations were compared, following the procedure of randomized complete block design. For the purpose of ANOVA in-house developed statistical packages were used. The standard deviations are graphically represented.

3. Results

3.1. Confirmation of toxicity of culture filtrate

Confirmation of toxicity of the culture filtrate of A. alternata was established by its inhibitory effects on root growth of the germinating wheat seedlings. The toxic concentrates also produced necrotic spots on the leaves of geranium 48 h after its application. The spots gradually enlarged and clear chlorotic zones formed around them after 72 h. It was thus established that symptoms induced by the filtrate on excised leaves were similar to those produced by the pathogen under the natural infection conditions. No such effect was observed on the leaves applied with un-inoculated V₈ medium or boiled culture filtrate incubated for 25 d.

3.2. Development of resistant callus cultures

It was observed that the calli retained regeneration ability on toxin-free medium 7 d after treatment. However after the 7-d incubation period, the survival rate of calli treated with culture filtrate was inversely proportional to the toxic dose (Fig. 1). The growth of the surviving calli pieces was inhibited by up to 40% as the concentration of filtrate increased from 0 to 20%.

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toxin-treated calli, on semi-solid medium was slow and proportional to the culture filtrate concentration applied. The frequency of survival of calli at 0–16% of the culture filtrate was 100%, 86.7%, 53.3% and 13.3% respectively while that on 20% was nil (Fig. 1).

3.3. Regeneration response of toxin-treated calli

The surviving calli on transfer to the toxin-free medium started regenerating after 10 d to significantly different extents (Fig. 2A–E). The maximum frequency of regeneration that was same as that of control calli was noted with a 4% dose of culture filtrate. There was little or no regeneration exhibited by the calli exposed to 16% and 20% levels of CF. The regenerated plants derived from calli surviving the 16% concentration treatment ultimately failed to proliferate further and died within 6 d. The number of regenerants was higher in the calli treated with 8% CF than in 12% (Fig. 1). A negative relation could be observed between the toxin concentration and the following parameters: number and length of shoots, number of leaves borne on the plantlet and size of root system formed. The regenerants obtained from calli treated with 4% CF were almost identical to the control plants with respect to

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Fig. 2. Regeneration response in the callus tissue of Pelargonium graveolens cv. Hemanti raised with and without pre-selection treatment of Alternaria alternata culture filtrate. (A) Regeneration in untreated control callus; (B–E) varying degrees of regeneration response in toxin-treated calli (B—8%, C—12%, D—16% and E—20% culture filtrate).
different parameters tested and hence were not considered further. Alteration in growth behaviour was noted in plants derived from calli treated with 12% CF; these were smaller and had smaller root systems. Even though differences were not statistically significant, the regenerants obtained from 8% CF-treated calli appeared healthier than the plants obtained from 12% CF-treated calli (Fig. 3).

3.4. Phytotoxicity bioassay

During the next phase of experimentation, leaves from the regenerants of resistant calli were subjected to a phytotoxicity test, where the leaves were cultured on semi-solid MS medium supplemented with 8%, 12%, 16% and 20% (v/v) of CF. The 4% dose of CF, which was not effective in the selection of resistant calli, was not applied. The excised leaves of regenerants that had developed from the CF-treated calli at 8% and 12% exhibited resistance on the toxic media in contrast to the leaves of control plants (Fig. 4A–C). They remained green when placed on media containing 0–12% of CF but could not tolerate media containing higher levels of CF (16–20%) (Fig. 4D–E). The leaves surviving the phytotoxicity test at 8% and 12% CF when transferred to regeneration medium proliferated shoots (Fig. 4F).

3.5. In vivo testing of regenerated plants against the pathogen

The pathogen resistance of individual plants was scored for the severity of infection. The cuttings from the control and 4% CF-treated calli were found to be susceptible, while those from 8% and 12% CF-treated calli were moderately resistant and resistant, respectively (Fig. 5).

4. Discussion

In the present work, the callus cultures produced variants from which the resistant regenerants to both the fungal pathogen and its culture filtrate could be obtained. A number of investigations have obtained success in selecting resistant materials using culture filtrates of various pathogens like *Phoma lingam*, *Xanthomonas campestris* pv. *Pruni*, *Fusarium oxysporum* f. sp. *gladioli* (Mass.), *Fusarium solani*, *Phytophthora capsici*, etc. (Sacristan, 1982; Hammerschlag, 1988; Remotti et al., 1997; Hartman et al., 2004; Ping and Mu, 2004).

Culture filtrates in several cases have shown the presence of extracellular non-specific phytotoxins that determine pathogenicity (Nachimas et al., 1979; Pennisi et al., 1988; Sesto et al., 1990). Also, a correlation is a must between the *in vitro* and *in vivo* responses of the host to the infection caused by a pathogen for the toxin to be utilized for the selection of disease-resistant genotypes. In the present study too, the appearance of necrotic spots followed by chlorosis of the leaf after the application of a culture filtrate of *A. alternata* confirmed that there is some factor present in the CF which is responsible for causing disease symptoms similar to those caused by the pathogen in natural infection conditions.

In accordance with the noted fact that toxins are, very likely, poorly transported in the callus and that survivability of the callus in toxic medium is dependent on its size (Behnke, 1979), a standard size of about 1 mm² was found ideal in order to obtain reproducible results and at the same time facilitating exposure of the maximum surface area of the calli to the toxic medium. Furthermore, in contrast to an earlier published report (Buiatti et al., 1985) in the course of the present investigation, liquid medium was preferable to solid in maximizing the penetration and transportation of the toxic media into the cells.

In accordance with the published report (Ling et al., 1985), the growth of the selected surviving calli on the toxin-free medium was slow and the degree of growth inhibition was directly proportional to the concentration of culture filtrate added to the medium. The inhibitory effect of the culture filtrate of *P. lingam* on callus in *Brassica napus* has also been noted (Sacristan, 1982). A phytotoxicity test was carried out using leaves of these *in vitro*-raised regenerants to confirm whether these regenerants from resistant calli possessed sensitivity to the pathogen or in other words carried resistance against the culture filtrate of pathogen.

Almost all the regenerants exhibited resistance to *A. alternata in vivo*, whereas none of the control plants showed resistance. This establishes a strong correlation between *in vitro* selection and *in vivo* resistance as has already been observed in alfalfa (Arcioni et al., 1987), tomato (Shahin and Spivey, 1986) and *Dianthus* (Thakur et al., 2002). It appears that the development of resistance in callus cultures of *Pelargonium graveolens* cv. Hemanti exposed to a fungal culture filtrate of *A. alternata* is the first report of its kind.

The development of resistance *in vitro* in callus cultures against the phytotoxin of a particular pathogen has been...
correlated with the secretion of extracellular proteins by the host in culture, which may be antifungal hydrolases or pathogenesis-related proteins (Legrand et al., 1987; Mauch et al., 1988; Jayasankar and Litz, 1998). It appears that calli raised from a variety of diploids and polyploids harbour considerable variation (Hansen et al., 2007). This variation must be responsible for the somaclonal variation encountered in plants regenerated from calli (Larkin and Scowcroft, 1981). The callus tissue seems to offer opportunities for exercising a variety of selection, including that for tolerance to phytotoxins. This may allow recovery of all kinds of desired variants, related to recessive or dominant genetic changes.

The present experiments on rose-scented geranium have been successful in opening the doors for developing disease resistance by the selection of resistant callus cultures against fungal culture filtrate. This approach could possibly be extended to other diseases of geranium. It may even be possible to select for resistance to toxic extracts of several disease-causing organisms.
Fig. 5. Resistant (A) and control (B) plants 15 d after inoculation with A. alternata.

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