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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 1 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.

Keywords: Fungal culture filtrate; Disease resistance; Fungal pathogen; Leaf blight disease

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.

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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% $HgCl_2$ 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} α-naphthaleneacetic 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 mm^2 pieces of the fungal mycelium in 1 l Roux bottles containing 100 ml of liquid V_8 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 mm^2 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).