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In vitro conservation of pointed gourd (*Trichosanthes dioica*) germplasm through slow-growth shoot cultures: Effect of flurprimidol and triiodobenzoic acid

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ABSTRACT

The effects of growth retardants, flurprimidol and triiodobenzoic acid (TIBA) were investigated on the development of slow-growth cultures aimed for *in vitro* conservation of pointed gourd (*Trichosanthes dioica*) germplasm. Shoot cultures maintained on MS medium supplemented with flurprimidol (1.0, 2.0, 5.0, 7.5, 8.8, 10.0, 12.5, 15.0, 20.0 μM) or TIBA (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M) were stored at 25 °C and 9 °C. In general, flurprimidol in the medium suppressed shoot growth, but it promoted leaf (up to 5.0 μM) and root development (all the tested concentrations). The optimum concentration of flurprimidol for storage of the cultures was 7.5 μM as it supported culture survival for 784 days, when stored at 9 °C. In general, TIBA promoted shoot growth and number of leaves per culture at low concentrations (10^{-8} M), but it had suppressive effect at the higher concentrations. However, root number per culture decreased significantly in response to even 10^{-8} M TIBA. The shoot cultures maintained on medium containing 10^{-8} M TIBA survived for 280 d, while the cultures grown on GR-free MS medium and stored at 9 °C survived much longer (~700 d). Use of TIBA for slow-growth of pointed gourd shoot cultures did not offered any advantage over the GR-free MS medium. The cultures stored at 9 °C survived much longer than those stored at 25 °C. The GR-free MS medium may be preferable to the 7.5 μM fluprimidol as it supported culture survival for up to 700 d at 9 °C, and avoids the additional cost and the possible undesirable physiological and genetic effects of the growth retardant. But when low temperature storage is not feasible, the cultures may be maintained on MS medium supplemented with flurprimidol (5.0 or 7.5 μM) as the cultures survived up to 364 d at 25 °C.

1. Introduction

Pointed gourd (*Trichosanthes dioica* Roxb.) is a dioecious, perennial cucurbit vegetable grown in several states of India, and in some other Asian countries. Its fruits are used as vegetable, and are liked by consumers for their easy digestibility and some medicinal properties recognized by the indigenous systems of medicine (Sharma and Pant, 1988). It produces many seeds per fruit, but the seeds normally fail to germinate even after scarification and some other treatments (Kumar et al., 2008). In view of this, and the fact that it is highly heterozygous due to the dioecious nature, it is commercially propagated by stem cuttings (Singh, 1989). This propagation method is not only slow, but it also runs the risk of propagating the infections by pathogens. Therefore, efforts have been made to develop micro-propagation protocols using nodal segments for axillary shoot proliferation (Abdul-Awal et al., 2005; Malek et al., 2007; Kumar, 2008), as well as cotyledon explants (Hoque et al., 1998) for regeneration of shoots. *In vitro*-raised shoots are readily rooted, and the plantlets can be established in the field with a high survival frequency (Hoque et al., 1998; Mythili and Thomas, 1999; Kumar, 2008).

The germplasm of pointed gourd is traditionally conserved in the field gene banks, which is a time and labor intensive task. In addition, field gene banks are always prone to insect pest and pathogen attack, and damage by animals and natural hazards (Engelmann, 2004). Under such conditions, *in vitro* approaches, *viz.*, cryopreservation and slow-growth shoot cultures assume significance for the *in vitro* conservation of pointed genetic resources. The *in vitro* conservation techniques could be useful to overcome the problems of dormancy, manpower requirements and need for large field areas. Such methods also allow maintenance of elite lines, transgenic materials, and mutant lines till their field establishment or release approval (Suprasanna et al., 2011). For the last

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ten years, compared to classical cryopreservation techniques, new advancements have been made primarily based on vitrification, and encapsulation–dehydration, which have been applied to a broad range of species, but their routine use is still limited (Gonzalez-Benito et al., 2004; Engelmann, 2004). In general, cryopreservation requires considerable technical sophistication, relatively expensive equipments, continuous supply of liquid nitrogen, and involves substantial running expenditure (Bhojwani and Razdan, 1996; Engelmann, 2004). In contrast, slow-growth shoot cultures can be maintained either in a regular tissue culture room or in a chamber maintained at a low temperature (usually, 4–15 °C). Both of the above approaches are being used for conservation of germplasm of important asexually propagated species by institutions like International Center for Potato, Lima, Peru and National Bureau of Plant Genetic Resources, New Delhi, India (Singh, 2012). Although, a number of reports are available on the regeneration and high frequency multiplication of pointed gourd *in vitro* (Abdul-Awal et al., 2005; Malek et al., 2007; Kumar, 2008; Hoque et al., 1998) but no report is available on development of slow-growth shoot cultures for medium-term conservation of this very important vegetable species.

This study was undertaken with a view to develop a suitable slow-growth shoot culture technique for *in vitro* conservation of pointed gourd germplasm. The culture medium was supplemented with different concentrations of the growth retardants, flurprimidol and triiodobenzoic acid (TIBA) and stored at two temperatures (9 °C and 25 °C) with a view to induce slow-growth. Flurprimidol is similar to ancymidol, and both inhibit gibberellin biosynthesis by blocking the conversion of ent-kaurene to ent-kaurenoic acid (Rademacher, 2000). However, flurprimidol is reported to be two to four times as active as ancymidol and is more stable than the latter.

2. Materials and methods

2.1. Plant materials, culture medium and conditions

All the laboratory experiments were conducted at Plant Biotechnology Laboratory, School of Biotechnology, Banaras Hindu University, Varanasi. Young vines (30–40 cm) were collected from field-grown plants of *T. dioica* female clone IIVRPG-102. Stem segments of 6.0–8.0 cm containing 3–4 nodes were washed thoroughly in running tap water and then treated with 1.0% (w/v) cetrimide solution for 30 min. After thorough rinse in running tap water, the explants were treated with a solution containing an antibiotic (trimethoprim; 0.05%) and a fungicide (dimethyl carbendazim, 0.2%) for 2 h. Explants were surface sterilized using $HgCl_2$ (0.1%) for 3 min followed by 4–5 rinses with sterile distilled water. In the end, single-node explants of ~1.5 cm were inoculated onto MS (Murashige and Skoog, 1962) basal or MS half-strength medium for culture establishment. In this study, cultures kept in 25 mm × 15 mm borosilicate glass culture tubes; each tube had 20 ml medium and a single explant was inoculated into each tube. The cultures were incubated at 25 ± 2 °C, under 16 h light period of white light from fluorescent tubes at unit of irradiance 50 E m^{-2} s^{-1} and 8 h dark period. These cultures were sub-cultured on MS medium containing 4.44 μM of 6-benzyladenine (BA) at every 4–5 weeks to multiply and serve as stock culture of nodal explants for the experiments on slow-growth.

2.2. Evaluation of flurprimidol and triiodobenzoic acid (TIBA)

The effects of the two inhibitors, *viz*., flurprimidol and TIBA, were evaluated in two separate experiments: in one experiment, ten different concentrations of flurprimidol (0.0, 1.0, 2.0, 5.0, 7.5, 8.8, 10.0, 12.5, 15.0 and 20.0 μM) were evaluated, while seven different concentrations of TIBA (0.0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M) were tested in the second experiment. Each experiment was conducted as per a three-level nested design with five replications. Two culture storage temperatures, *viz*., 25 ± 2 °C and 9 ± 2 °C, represented the first level of nesting, while the second level of nesting within each storage temperature comprised the different flurprimidol or TIBA concentrations. The third level of nesting was due to the culture storage duration at the time of recording of the observations. The different concentrations of flurprimidol or TIBA were added to the growth regulator (GR)-free MS medium. Nodal explants having one to two nodes each were excised from the stock cultures and placed on the different media to initiate shoot cultures. All the cultures were initially stored in the culture room at 25 ± 2 °C for 10 d. Thereafter, ten healthy cultures per inhibitor concentration were left in the same culture room while ten other cultures were shifted into a refrigerator maintained at the temperature of 9 ± 2 °C. Five of the cultures maintained at each concentration of flurprimidol or TIBA at each of the two temperature regimes (9 °C and 25 °C) were used for recording of the observations on the numbers of shoots, leaves and roots *etc*., and each culture was treated as a replicate. The cultures maintained on GR-free MS medium (with 0.0 flurprimidol/TIBA) and stored at 25 °C or 9 °C served as controls for the respective temperature regimes.

The remaining complete set of five cultures per flurprimidol/TIBA concentration stored at the 25 °C and 9 °C was left undisturbed till it was used for assessing culture survival. The culture survival was assayed when the cultures showed extensive senescence, *i.e*., nearly all the leaves and top halves of the shoots appeared dry and only the bottom halves of the shoots were visibly green. At this point of time, one to two-node explants were excised from the green parts of the shoots, cultured on MS medium supplemented with 4.44 μM BA, and incubated at 25 ± 2 °C. In the cases of cultures with relatively smaller shoots, the entire green portion of the shoot was used as explant. A culture was considered to be 'dead' when none of its nodal explants was able to support shoot proliferation. In case, three or more of the five cultures in a treatment were considered as 'dead', the treatment was recorded as lost. Four weeks after the survival assay, the remaining five cultures maintained on each treatment were evaluated for survival by culturing their nodal explants, but most of the cultures were dead by this time. The shoots recovered from these nodal cultures were tested for rooting and establishment in soil-filled pots.

In both the experiments (for evaluation of the two inhibitors), data were recorded on number of shoots per culture, shoot length (cm), number of leaves per shoot, and number of roots per shoot at the following intervals: 4, 16, 28, 40, 52, 64, 76, 88, 100 and 112 weeks after culture initiation in the case of flurprimidol and 4, 16, 28 and 40 weeks after culture storage in the case of TIBA. Since the cultures maintained on TIBA could not survive beyond 40 weeks, this experiment was terminated at this point of time.

2.3. Statistical analyses

The data for shoot length and the other two traits pertaining to the culture storage periods of up to 28 weeks (for flurprimidol) and up to 16 weeks for TIBA were subjected to analysis of variance (ANOVA; Gomez and Gomez, 1984) according to the three-level nested design with five replications. The effects of the inhibitor concentrations were nested within the two culture storage temperature regimes, while the effects of the storage periods were nested within each flurprimidol or TIBA concentration. The data from the later observations (beyond 28 weeks in the case of flurprimidol and beyond 16 weeks for TIBA) could not be analyzed as per nested design as there were many missing treatments due to the