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SHORT COMMUNICATION

EFFECT OF THIADIAZURON AND ASCORBIC ACID ON RECURRENT PRODUCTION OF PLANTLETS FROM SEEDS OF *SYZYGIUM ALTERNIFOLIUM* (WIGHT.) WALP

P.S. SHA VALLI KHAN[1,2], E. PRAKASH[3,4] AND E.S. MERU[3]

[1]Department of Botany, Yogi Vemana University, Vemanapuram, Kadapa-516 003, Andhra Pradesh, India
[2]Departmento SENFIMIZO, Faculta di Agararia, Universita degli Studi di Palerma, Viale delle Scienze 11, 90128, Palermo, Italy
[3]Biotechnology Research Centre, Tirupati, Andhra Pradesh, India
[4]Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, 35, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan ROC

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SUMMARY

The current experiments were undertaken to study the effect of thiadiazuron (TDZ) and ascorbic acid (AA) on recurrent production of plantlets from seeds of *Syzygium alternifolium* (Wight.) Walp. The multiple shoots were induced from embryonal axes of seeds cultured on basic culture medium (BCM) containing half strength Murashige and Skoog (MS) salts, B_5 vitamins, 2 mg/l glycine, 2% (w/v) sucrose, 0.8 % (w/v) agar, 1.0 mg/l TDZ and AA (1000 mg/l). Our findings suggested recurrent production of plantlets and their hardening process to obtain 70% survival rates. The tissue culture protocol can be used for *en masse* propagation and conservation of this important multipurpose fruit tree.

***Key words*: Ascorbic acid, *in vitro*, Myrtaceae, polyembryony, *Syzygium*, TDZ**

Syzygium alternifolium (Wight.) Walp, belongs to the family Myrtaceae, commonly known as 'Mogi', is naturally polyembryonic and rare taxa, endemic to natural forests of Tirumala hills in the Southern Part of India (Anonymous 1956). Tissue culture protocols for micropropagation of *S. alternifolium* using nodal explants from seedlings as well as adult tree have already been reported (Sha Valli Khan *et al.* 1997, 1999a). However, frequencies of plant regeneration systems using juvenile and adult explants were moderate and produced relatively few propagules. Thidiazuron (TDZ), substituted phenylurea (N-phenyl-1,2,3- thidiazol-5-ylurea) has been used to stimulate higher rates of multiple shoot induction in many woody tree species, including recalcitrant ones. The frequency of shoot regeneration ability declined markedly at higher concentrations of TDZ with stunted growth of regenerated shoots (Huetteman and Preece 1993). Similarly in our earlier study, we have reported high frequency shoots formation from seeds with suppressed shoot growth on TDZ-supplemented medium (Sha Valli Khan *et al.* 1999b). Hence, the current experiments were undertaken to investigate combined effect of ascorbic acid (AA) and TDZ for inducing normal multiple shoots from seed cultures of *S. alternifolium*.

Mature fruits of *S. alternifolium* were collected during May and June from trees growing on Tirumala hills of Chittoor district, Andhra Pradesh, India. Dicotyledonous seeds were taken out from fruits

*Corresponding author, E-mail:pssvkhan@yahoo.com; Present address: Prof. M.A. Germana, Departmento SENFIMIZO, Faculta di Agararia, Universita degli Studi di Palerma, Viale delle Scienze 11, 90128, Palermo, Italy

removing the outer thick layer, washed with running tap water and successively surface sterilized with 70% (v/v) ethanol for 2 minutes and 0.1% (w/v) aqueous mercuric chloride for 5 min. After through washing, disinfected seeds were inoculated onto basic culture medium (BCM) containing half strength Murashige and Skoog (1962), B_5 vitamins (Gamborg *et al.* 1968), 2 mgl^{-1} glycine, 2% (w/v) sucrose, 0.8% (w/v) agar, TDZ and ascorbic acid. Effect of TDZ in combination with ascorbic acid (1000 mgl^{-1}) tested in 0.0 mgl^{-1}, 0.5 mgl^{-1}, 1.0 mgl^{-1} and 2.0 mgl^{-1} concentrations on multiple shoot induction from seeds. The pH of the medium was adjusted to 5.8 before autoclaving at 121ºC and 1.05 kg cm^{-2} pressure for 20 min. All growth regulators were added to the sterile medium after filter sterilization. The medium was dispensed in 20 ml aliquots into 25 mm X 150 mm culture tubes. Further in another set of experiments, the small shoot clusters removed from seed cultures were transferred onto fresh BCM containing 1.0 mgl^{-1} TDZ and 1000 mgl^{-1} AA for shoot proliferation. The sub-culturing process was repeated for four more passages for recurrent production of shoots. Data on number of shoots and shoot lengths were collected after 6 weeks. The cultures were incubated in a culture room at air temperature of 25 ± 2 ºC with 65% relative humidity under 16 h photoperiod with irradiation of 50 µ Em^{-2} sec^{-1} photosynthetically active radiation (PAR).

Single shoots from shoot cultures of 1st passage to 5th passage were excised and transferred onto agar (8 % w/v) solidified half strength MS medium supplemented with 2% (w/v) sucrose and 1.0 mg/l indole-3-butyric acid (IBA) medium for rooting. Rooted shoots were transferred to small plastic teacups (120 ml volume) filled with sterile vermiculite and kept in a plant growth chamber (NK systems, Japan) at air temperature of 25 ± 2 ºC with 85% relative humidity under 16 h photoperiod with irradiation of 50 µ Em^{-2} sec^{-1} PAR for 2 weeks hardening. Afterwards primary hardened plantlets were transferred to larger black polythene bags (15 cm height) containing soil: farmyard manure (3:1) and finally maintained in the shade area of forest nursery. All the experiments were conducted with a minimum of twelve replicates per treatment and repeated three times. The results are expressed as a 'standard deviation of the mean' of three repeated experiments.

Seeds cultured on a BCM germinated after 14 days of incubation. Approximately 65-70% of seeds produced single seedling per seed while remaining portion (35-30%) of seeds developed either multiple seedlings (3-4 shoots) or only shoots or only roots upon germination. Formation of multiple seedlings from a seed upon germination is ascribed to the existence of polyembryony (Batygina and Vinogradova, 2007). The expression of less percentage of polyembryony than its actual frequency in mature seeds was attributed due to arrest of additional embryos at early stages or their degeneration during the course of seed development (Bhojwani and Bhatnagar 1984). The addition of TDZ and AA (1000 mgl^{-1}) to BCM was found to improve the percentage of seeds forming multiple shoots from embryonal axes (Fig.1). Of the various concentrations of TDZ in combination with AA (1000 mgl^{-1}) tested, 1.0 mgl^{-1} TDZ concentration yielded highest number of shoots per seed (32-33) with satisfied shoot length (3.5 cm) after 6 weeks of incubation. At higher concentration of TDZ (i.e.2.0 mgl^{-1}) average number of shoots as well as length evidently declined (Table 1). In our previous study TDZ and 250 mgl^{-1} AA has been found to be very efficient to induce clusters of a dense, compact mass of very small shoot buds measuring less than 0.5 cm in length in seeds of *S. alternifolium* cultured *in vitro* (Sha Valli Khan et al. 1999b). In the current experiment, the addition of 1000 mgl^{-1} AA into the 1.0 mgl^{-1} TDZ media combination induced multiple shoots from emryonal axes of seeds and compensated for the negative effects of TDZ by increasing the shoot growth approximately 7 times (from 0.5 cm to 3.5 cm) after 6 wk of culture.

Table 1. Effect of TDZ on induction of multiple shoots from seeds

TDZ-[a] (mgl^{-1})	Shoot number	Shoot length (cm)
0.0	2.6 ± 0.5	6.8 ± 0.4
0.5	12.5 ± 0.3	4.1 ± 0.7
1.0	32.5 ± 1.0	3.5 ± 0.1
2.0	24.5 ± 3.0	2.9 ± 0.3

Data was collected after 6 weeks of incubation.
Values represent means of three replicates of twelve seeds ± standard deviation
-[a] Medium supplemented with 1000 mg/l AA

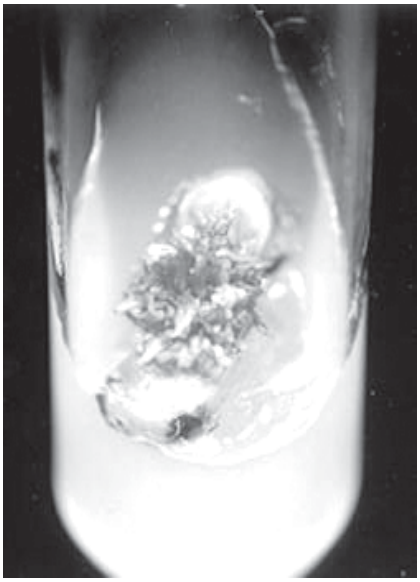

Fig. 1. Multiple shoot induction from seeds of *S. alternifolium* on MS medium supplemented with 1.0 mgl^{-1} TDZ and 1000 mgl^{-1} AA

Fig. 2. Shoot proliferation form axilary buds of small shoot cluster cultured on MS medium supplemented with 1.0 mgl^{-1} TDZ and 1000 mgl^{-1} AA

AA in combination with cytokinins was shown to play a key role on *in vitro* morphogenesis and plant regeneration in *Gladiolus* (Datta Gupta and Datta 2003). Ascorbate has also been reported to be an important cofactor for a number of enzymes involved in hormone synthesis, e. g., gibberellins (Prescott and John 1996). This opens yet another possible explanation for improved shoot elongation. Multiple shoots developed from the axillary buds of the shoots of small clusters on fresh medium supplemented with 1.0 mgl^{-1} TDZ and 1000 mgl^{-1} AA. The number of axillary shoots was increased to an average of 11 healthy, green shoots each having 2-3 cm length at the end of 5th passage of shoots multiplication (Fig. 2). The enhanced shoot multiplication by subsequent cultures substantiates the earlier reports on *S. alternifolium* (Sha Valli Khan *et al*. 1999a) and *S. cuminii* (Jain and Babbar 2000). Adopting this procedure, recurrent production of shoots can be achieved through repeated sub-culturing.

Rooting of the shoots was readily achieved when excised shoots were cultured on half strength of MS medium containing 1.0 mgl^{-1} IBA and this was similar to our previous finding (Sha Valli Khan *et al.* 1997, 1999a). *In vitro* formed shoots produced 10-12 white, thick roots having 2-3 cm length after 6 weeks of sub culturing. In the present study, percentage of rooting enhanced from 75 % shoots obtained from 1st passage to 92% of shoots obtained from the 5th passage of sub-culturing. After five months of establishment in soil, tissue cultured plantlets showed a high degree of uniformity without any detectable phenotypic variation. The present finding establishes a protocol for the use of TDZ as a potent cytokinin for recurrent production of plantlets from seed cultures of *S. alternifolium.* The procedure can be used for *en masse* propagation and conservation of this important multipurpose fruit tree.

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