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Effect of Amino Acids and Growth Regulators on Indirect Organogenesis in *Artemisia vulgaris* L.

S. Pradeep Kumar and B.D. Ranjitha Kumari
Stress Physiology and Medicinal Plant Biotechnology, Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India

Abstract: *Artemisia vulgaris* L. (mugwort) belongs to the family Asteraceae and is a tall aromatic perennial herb. Mugwort contains volatile oils, sesquiterpene lactones and flavonoids used for insecticidal, antimicrobial and antiparasitical properties. All parts of the plant are used for antihelmintic, antiseptic, antispasmodic, carminative, cholagogue, digestive, expectorant, nervine, purgative and stimulant. This study describes the effect of amino acids and growth regulators on callus induction, multiple shoot and root induction using cotyledonary explants. Murashige and Skoog medium supplemented with B5 vitamins containing 2, 4D, NAA and cysteine combination was found better response for callus induction. Maximum number of multiple shoots (95.1%) per explants after 20 days of culture, with combination of BA, TDZ and tyrosine was found better response in the medium. NAA, $AgNO_3$ and glutamine combination produced maximum number (98.7%) of roots per explants in the medium. Plants produced from *de novo* regeneration on excised tissues will be useful for crop improvement through genetic engineering and cell culture techniques.

Key words: Asteraceae, glutamine, mugwort, multiple shoots, phytohormones, silver nitrate

INTRODUCTION

Artemisia vulgaris L. (mugwort) belongs to the family Asteraceae and is a tall aromatic perennial herb. Mugwort contains volatile oils, sesquiterpene lactones and flavonoids used for insecticidal, antimicrobial and antiparasitical properties. In traditional medicine, this plant is being widely used for the treatment of diabetes, epilepsy, depression insomnia and anxiety stress (Walter *et al*., 2003).

All parts of the plant are antihelmintic, antiseptic, antispasmodic, carminative, cholagogue, digestive, expectorant, nervine, purgative and stimulant. The essential oils of the plant were reported to exhibit 90% mosquito repellency against *Aedes aegypti*, a mosquito that transmits yellow fever (Ram and Mehrotra, 1995). A paste or powder of the leaves is applied over skin diseases (Kapoor, 2000). In recent years, there has been an increased interest in *in vitro* techniques, which offers powerful tools for germplasm conservation and the mass multiplication of many threatened plant species (Murch *et al*., 2006). *In vitro* micropropagation using shoot tips (Arditti and Ernst, 1993), stem nodal

Corresponding Author: B.D. Ranjitha Kumari, Stress Physiology and Medicinal Plant Biotechnology, Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India
Tel: 91-0431-2407061 Fax: 91-0431-2407045

segments (Nayak *et al*., 1997) and root tips (Park *et al*., 2003) has been successfully used for propagation of a number of orchids either for conservation or for commercial production. Direct regeneration of multiple shoots without an intermediate callus phase shortens the duration for regeneration and reduces the likelihood of incidence of Somaclonal variation (Polonca *et al*., 2004). Dual phase or liquid medium overlay culture technique is a novel approach in plant tissue culture which improves the rate of regeneration and development (Visure, 1985; Thomson *et al*., 2007; Pullman and Skryabina, 2007; Sim *et al*., 2007). Such a technique involves the use of an agar solidified medium overlaid with a liquid medium fraction. This technique has been shown to have certain advantage over the use of conventional agar solidified medium or purely liquid medium. Induction of multiple shoots in a monopodial orchid hybrid (*Aerides vandarum* Reichb.f x *Vanda stangeana* Reichb.f) using thidiazuron and analysis of their genetic stability (Kishor and Devi, 2009). Besides development of a novel micropropagation system, an efficient growth regulator capable of inducing tremendous organogenesis from different explants is necessary. Thidiazuron (TDZ: N-phenyl-N0-[(1, 2, 3-thidiazol-5-yl)urea]), a non-purine cytokinin compound, has been shown to exhibit a stronger effect than N6-benzyladenine (BA) on *in vitro* morphogenesis of a wide range of crops (Malik and Saxena, 1992; Nayak *et al*., 1997; Vinocur *et al*., 2000; Park *et al*., 2003; Sujatha and Kumari, 2007).

Exogenously added amino acids play an important role in plant tissue culture but culture media of existing regeneration protocols are scarcely supplemented with amino acids. Effect of various amino acids on shoot regeneration of sugarcane *Sacchrum officinarum* L. (Asad *et al*., 2009). Specific media components involving amino acids have been found to play an important role on tissue culture systems of certain species (Benson, 2000). Amino acids have been used as organic nitrogen source in *in vitro* cultures of several species as alfalfa, maize, sorghum, pineapple, rice and other monocots to enhance somatic embryogenesis and regeneration (Skokut *et al*., 1985; Claparols *et al*., 1993; Rao *et al*., 1995; Hamasaki *et al*., 2005; Grewal *et al*., 2006). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon, 2007). Therefore, the objective of the present study was to conduct detailed and systematic studies on three different amino acids to determine optimum amino acid concentration to develop the most efficient *Artemisia vulgaris* regeneration system. Three different amino acids, glutamine, cysteine and tyrosine were evaluated for their ability to induce callus, multiple shoots and root induction in *Artemisia vulgaris*. To our knowledge, this is the first report in which a number of different amino acids have been compared for their effects on *Artemisia vulgaris* regeneration.

MATERIALS AND METHODS

Plant Material, Culture Medium and Culture Conditions

Artemisia vulgaris L. seeds were collected from Johnny's selected seeds, USA located at Winslow, Maine during the year July 2007-2008. *In vitro* seed germination was carried out and seed sterilization processes were described previously by Sujatha and Kumari (2007). Surface sterilized seeds were inoculated Murashige and Skoog (1962) germination (MSG) medium. Cultures were initially incubated in darkness for 5-7 days at a temperature of 23°C to facilitate germination. Later, they were transferred to photoperiodic conditions and maintained for another 28-30 days for seedling growth.

In Direct Organogenesis and Plantlet Regeneration

Callus Induction

Calli were induced from cotyledonary nodal explants excised from 30-d-old seedlings grown on MS medium containing B5 vitamins, 2% (w/v) sucrose, 0.8% (w/v) Difco Bacto Agar (Hi-media, Mumbai, India) and supplemented with various concentrations (0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}) of each of 2, 4-dichlorophenoxyacetic acid (2, 4-D), α-Naphthalene acetic acid (NAA) and cysteine (cys). With controls without additional amino acids, all the experiments were repeated three times and completely randomized design experiment was conducted with three replicates per treatment. The frequency of explant forming embryogenic callus, color and texture were recorded after 6-9 weeks of culture. After 5-7 days of callus initiation, the primary tiny calli of explants were separated aseptically from the source of explants, so that no contact of parental tissue remained and set them again on the same medium for proliferation of calli without root-shoot differentiation. Repeated sub-culturing was done after every two weeks for maintenance and proliferation of the calli. The quantitative measurement of callus growth was estimated in terms of percentage of callus.

Multiple Shoot Induction

Cotyledonary nodal explants cuttings (1 cm long) were inserted either vertically, with 2±3 mm of the cutting inserted into the medium and the apical 7±8 mm protruding, or horizontally on the surface of the culture medium, containing B5 vitamins with various concentrations (0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}) BA, TDZ and Tyrosine (Tys) combination with controls without additional amino acids. At the end of the incubation period, the number of shoots which developed was recorded separately for each cutting end. All Plant Growth Regulators (PGRs) were purchased from Sigma (St. Louis, MO, USA). The pH of the medium was adjusted to 5.7 with 1 N NaOH or HCl and autoclaved at 121°C and 1.05 kg cm^{-2} pressure for 20 min.

Rooting and Transplantation

To induce rooting, individual elongated shoots (5-7 mm) in length were excised and Cultured for 60 day on 10 mL of semi-solid medium containing different types of auxins and amino acid viz., α-Naphthalene acetic acid (NAA) (0.5-3.0 mg L^{-1}) silver nitrate ($AgNO_3$) (0.5-1.5 mg L^{-1}) and glutamine (Gln) (0.5-1.0 mg L^{-1}) individually. One set of the cultures were inoculated in basal MS medium without the addition of amino acid and kept as control. The regenerated plants with well developed sufficient root system were ready for transfer to soil. When the plantlets remained in culture they were brought out of the controlled environment of growth room and were kept in the room temperature for 2-3 days to bring then in the contact of normal temperature. The plantlets were then rescued very carefully from the culture tubes. Agar attached to the root was washed gently under running tap water. Immediately after that they were transplanted to small pots containing sterilized ground soil, sands and cow dung in the ratio of 1:2:1. The pots with plantlets were kept in shade place and necessary cultured management was undertaken for good growth and development of the plant. After 7 days, the pots with plants were transferred to direct sunlight and after 15-25 days plantlets were finally transferred in new pots.

Statistical Analysis

Slide write software was used for graphical presentation of data and test of significance was carried out using analysis of variance. Treatment means were separated using the Duncan's Multiple Range Test (PROC GLM, SAS Institute, 1996).

Data were tested by ANOVA and level of significance (Gomez and Gomez, 1976).

RESULTS

Callus Induction

Callus was induced from cotyledonary explants on MS medium containing 2, 4-D, NAA and cysteine (cys) combination (1.5+1.0+0.5 mg L^{-1}) was found most effective for callus induction when compared with 2, 4-D and NAA (2.0+1.0 mg L^{-1}) (Table 1). It produced 72.4, 83.3, 96.7 and 90.6% callus respectively in 2, 4-D, NAA and Cysteine combination, when compare to 2, 4-D, NAA produced (67.8, 76.6, 89.3 and 83.1%) (Fig. 1a, b). The nature of cotyledonary nodal explants derived calli were yellows compact but fragile and tends to be very dry with increasing concentration of 2, 4-D.

Effect of Growth Regulators on Indirect Multiple Shoot Induction

The effect of growth regulators on shoot formation at the basal end of Cotyledonary nodal explant cuttings inserted in the medium supplement with BA, TDZ and Tyrosine (Tys) combination (2.0+1.0+0.5 mg L^{-1}) was found most effective for development of multiple shoots when compared to BA, TDZ in the medium (Fig. 2a-f). It produced (70.8, 79.4, 97.7 and 88.6%) of multiple shoots, respectively. Where as BAA and TDZ produced (59.5, 66.8, 79.9 and 74.5%) (Fig. 3a, b). Similar result was also reported in *Artemisia vulgaris* L. by Sujatha and Kumari (2007) but it is in stem and node explants. Frequency of shoot induction was observed to be more in 2-3 weeks of incubation. In three different modes of Amino acids supplementation the experiment was carried out (Table 2).

Rooting and Establishment in Soil

A proportion of the regenerated shoots rooted without the addition of an auxins to the medium (Table 2). The rooting frequency was higher in the medium supplemented with

Table 1: Effect of growth regulators on callus induction of *in vitro* raised Cotyledonary Nodal Explants of *A. vulgaris* L.

Plant growth regulators (mg L^{-1})	No. of callus per explants (Cotyledonary)	Callus induction % of response
Control	0.0	0.0
2,4-D+NAA		
0.5+0.5	6.4d	67.8d
1.0+0.5	7.3c	76.6cd
2.0+1.0	8.7a	89.3a
2.5+1.5	8.1bc	83.1b
2,4D+NAA+Cys		
0.5+0.5+0.5	7.1d	72.4d
1.0+0.5+0.5	8.2c	83.3c
1.5+1.0+0.5	9.7ab	96.7a
2.0+1.5+1.0	9.1b	90.6ba

Evaluation was made after 2 weeks of culture. Treatment means followed by different letters are significantly different from each other at 5% levels of significance ($p \leq 0.05$) according to DMRT

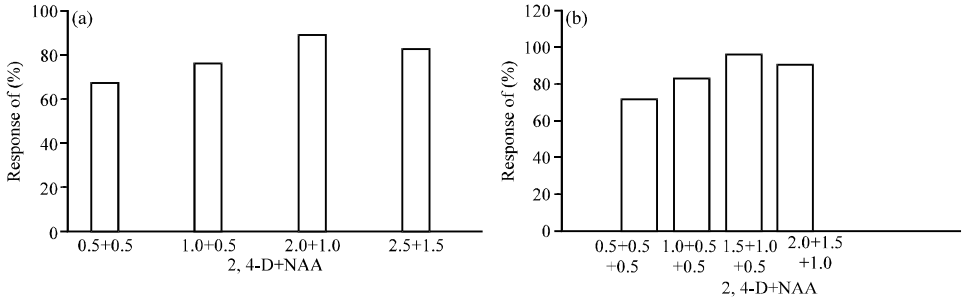

Fig. 1: Callus induction percentage of growth response

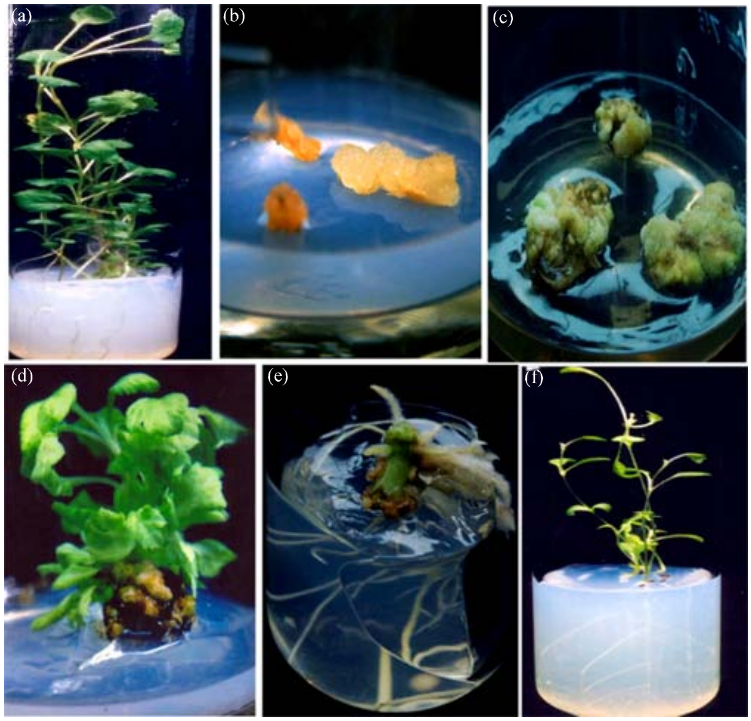

Fig. 2: Effect of various combinations of growth regulators and amino acids on in Direct Organogenesis in *Artemisia vulgaris* L. (a) *In vitro* seed germination, (b) fragile callus induction, (c) compact callus induction, (d) indirect multiple shoot, (e) rooting from cotyledonary node and (f) elongated shoots with roots

Table 2: Effect of various concentrations (mg L^{-1}) and combinations of growth regulators and amino acids on regeneration of shoot and root induction from cotyledonary nodal explants

Plant growth regulators (mg L^{-1})	Multiple shoots per explants (Cotyledonary)	Response (%)	No. of roots per explants (Cotyledonary)	Response (%)
Control	0	0	0	0
BAP+TDZ				
0.5+0.5	16.4d	59.5d	6.7cd	42.6cd
1.0+0.5	17.5cd	66.8c	6.5d	46.2c
2.0+1.0	19.3a	79.9a	11.5b	52.4b
3.0+1.5	18.9b	74.5ba	12.6a	54.7a
BAP+TDZ+Tys				
0.5+0.5+0.5	17.3d	70.8d	5.9d	43.2d
1.0+0.5+0.5	20.2c	79.4cd	11.6cb	47.6cd
2.0+1.0+0.5	23.4a	97.7a	12.8b	54.7b
2.5+1.5+0.5	20.3b	88.6b	13.4a	59.4a
NAA+AgNo3				
0.5+0.5	10.2d	43.2d	19.1d	77.3dc
1.0+0.5	11.6c	48.2cd	19.5cd	81.5c
2.0+1.0	13.5a	53.6b	20.2a	85.3a
2.5+1.5	14.1a	57.8a	19.8b	80.6b
NAA+AgNo3+Gln				
0.5+0.5+0.5	10.7d	44.9d	20.4d	89.2d
1.0+0.5+0.5	12.3c	51.7cd	21.7c	91.8cd
2.0+1.0+0.5	13.9c	56.2b	24.1a	98.7a
3.0+1.5+1.0	15.3a	61.3a	22.2bc	93.1b

Evaluation was made after 3 weeks of culture Treatment means followed by different letters are significantly different from each other at 5% levels of significance ($p \leq 0.05$) according to DMRT

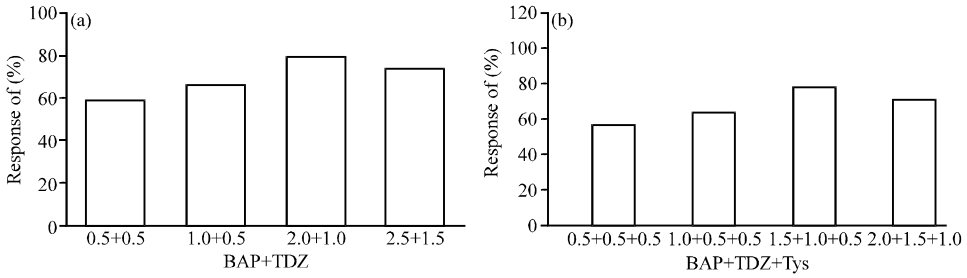

Fig. 3: (a, b) Multiple shoot induction percentage of growth

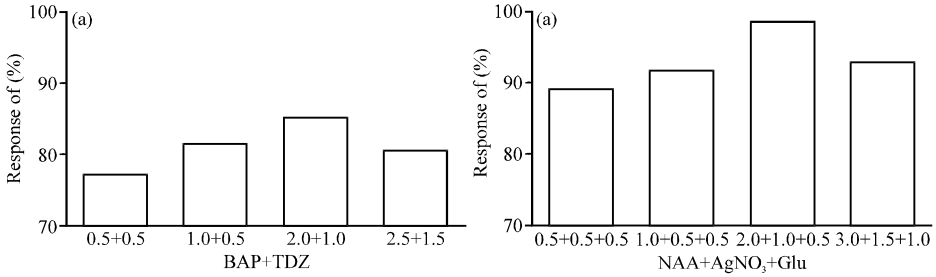

Fig. 4: (a, b) Rooting induction percentage of growth response

$NAA+AgNO_3$+Glutamine (2.0+1.0+0.5 mg L^{-1}) combination when compare to $NAA+AgNO_3$ (2.0+1.0 mg L^{-1}) in the medium. The percentage of rooting was high in $NAA+AgNO_3$+Glutamine (89.2+91.8+98.7+93.1%) (Fig. 4a, b) combination, when compare to $NAA+AgNO_3$ (77.3+81.5+85.3+80.6%) in the medium. A control group was also maintained. Plantlets significantly developed lengthy roots and root induction was strengthened within 15 days of culture. After 2 weeks, plantlets developed primary and secondary root system (Fig. 2). Frequency of rhizogenesis was almost 98%.

The successfully rooted plantlets were transferred to plastic cups containing sterile garden soil, farmyard soil and sand (2:1:1) for hardening plantlets were maintained in the culture room (25±1°C) conditions initially for 4 weeks and after transferred to normal laboratory conditions and maintained for about 1 week. Finally the plantlets were transferred to Botanical Evaluation Garden and maintained. The survival rate decreased to 98.3 and 80.7%, respectively after 5-6 weeks. There was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics. All the micropropagated plants were free from external defects.

DISCUSSION

Amino acids have been found critical to induce somatic embryogenesis in plant tissue culture medium. In orchard grass, embryos formed on amino acid containing medium showed high percentage of conversion and considerably less incidence of precocious germination (Trigiano *et al*., 1992). *In vitro* regeneration of multiple shoots directly from well developed seedlings of monopodial vandaceous orchids is a novel technique for their clonal propagation. Such a technique was reported earlier by Thomas and Michael (2007) and Kishor and Sharma (2008) using agar solidified medium. The main effect of BAP, TDZ and Tyrosine on Indirect regeneration in *Artemisia vulgaris* multiple shoot production was

evaluated after 2 weeks, when compare to previous study conducted by Kishor and Devi (2009) and also Sujatha and Kumari (2007). It was observed that type of amino acids and amount used in the medium had significant effect on the induction of multiple shoot. Tyrosine promoted maximum shoots production among the tested amino acids at 0.5 mg L^{-1}, while other non-amino acid treatments induced fewer shoots and the number of shoots developed increases with concentration up to at least 3.0 mg L^{-1}.

The few reports in which this pathway of regeneration has presumably been studied show a similar response (Goh *et al*., 1995) reported that some direct shoot regeneration occurred in the upper cut end of epicotyl segments of *Citrus grandis* on basal medium without added hormones. The number of multiple shoots induced by the present technique was approximately twice more than that obtained by Thomas and Michael (2007) for the monopodial orchid Rhyncho- stylis retusa (8.4 shoots per seedling) when it was cultured on agar solidified half-strength MS medium supplemented with 4.0 L M (2 mg L^{-1}) TDZ. So far, as the effectiveness of TDZ over benzyladenine (BA) in the induction of multiple shoot is concerned, Sujatha and Kumari (2007) obtained a two-fold increase in average number of shoots by 4.5 μmo L^{-1} (2 mg L^{-1}) TDZ over 4.4 μmo L^{-1} (2 mg L^{-1}) BA on multiple shoot induction in *Artemisia vulgaris in vitro* but these results were in stem and nodal explants only. Vinocur *et al*. (2000) also reported the superiority of TDZ over BA on shoot regeneration of root explants of Populus tremula and found that TDZ had exerted a 10-fold increase in the number of regenerated shoots.

Maximum percentage of root formation (Fig. 2) from cotyledonary nodal (98.7%) was observed on medium supplemented with NAA+$AgNO_3$+Glutamine. This result is in agreement with the findings of Dhar *et al*. (2000) on Pittosporum napaulensis and Agrawal and Sardar (2006) on Cassia angustifolia, where IBA was found better than NAA and IAA to induce the formation of maximum number of roots. The promotion of rooting from callus by IBA has also been reported in many other plant species (Saritha *et al*., 2002; Soniya and Das, 2002; Soniya and Sujitha, 2006). The overall objective of the current study was to develop a system for the mass propagation and aseptic growth of *A.vulgaris* plantlets derived from intact seedling system can be a prolific tissue for the biochemical characterization of medicinally active components and for the selection and cloning of superior individual genotypes. Plants produced from *de novo regeneration* on excised tissues will be useful for crop improvement through genetic engineering and cell culture techniques. These potential use of amino acids in promoting regeneration in other plant species could be applied in commercial propagation of elite cultivars.

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