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Research Article

IN VITRO PLANT REGENERATION OF CRITICALLY ENDANGERED MEDICINAL PLANT *ARTEMISIA AMYGDALINA* D.

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ABSTRACT
An efficient *in vitro* propagation protocol for *Artemisia amygdalina* D. developed here resulted in the induction of significantly more number of shoots (36 shoots per nodal explant) on a medium containing 10 µM benzyl amino purine (BAP) and 10 µM α-naphthaleneacetic acid (NAA) than any other treatment. Growth of the regenerated shoots in phytohormone free MS medium resulted in a significant increase in biomass, shoot height and shoot multiplication. The regenerated shoots also formed roots when transferred onto full strength and half strength MS (Murashige and Skoog) medium. Healthy *in vitro* raised plantlets were acclimatized and maintained in standard greenhouse conditions for further growth. This study provides a basis for germplasm conservation and further investigation of this rare, medicinally important plant species.
Keywords: *Artemisia amygdalina*, Kashmir, Tissue culture, Germplasm.

INTRODUCTION

Artemisia amygdalina D. is an endemic medicinal plant of Kashmir valley belonging to the family Astereacea, and grows in sub-alpine region of Kashmir Himalaya and North West frontier provinces of Pakistan. The plant extract is used locally for the treatment of epilepsy, piles, nervous disorders, cough, cold, fever and pain. The women folk of the valley use it for amenorrhea and dysmenorrhoea[1,2]. The major active principles in this plant are the terpenes, p-cymene, and 1, 8-cineole[3]. As a consequence of over-harvest and deforestation this plant is considered as the critically endangered endemic species of Kashmir valley[4]. Conventional propagation through seeds is difficult due to the high mortality of seedlings in early stages, further the use of rhizome for vegetative propagation seems not to be feasible due to the plant's endangered status. Tissue culture offers the possibility of rapid shoot proliferation without any harm to the plant. Several tissue culture systems have been developed with the aim to reduce production costs while maximizing plant growth[5,6]. *In vitro* propagation methods offer highly efficient tools for germplasm conservation and mass multiplication of many threatened plant species[7,8]. However, sustained supply to replenish the dwindling populations and the scientific research into the biochemical and medicinal efficacy of *A. amygdalina* D. requires a rapid, large-scale system for its production. In this communication, we report an efficient procedure for *in vitro* propagation of *A. amygdalina* D. for facilitating germplasm conservation efforts of this endangered medicinal plant species.

MATERIAL AND METHODS

Collection of plant material

The plant material was collected from Kashmir University botanical garden (KUBG) and identified in the Centre of Plant Taxonomy, Department of Botany, University of Kashmir under the voucher no. 1803 KASH.

Experimentation

Node explants were excised from 45-day old plants. The explants were surface sterilized by dipping in 70 % alcohol, then immersed in 10 % NaOCl for 20 minutes, followed by three rinses with sterile distilled water. Surface sterilized node explants (approximately 1 cm) were inoculated on MS medium[9]. Varying levels of BAP (2.5, 5, 10 and 15 µM) in combination with different levels of NAA (2.5, 5, 10 and 15 µM) were supplemented to the MS medium for shoot induction. The regenerated shoots were sub-cultured on MS basal medium for proliferation and elongation. All cultures were maintained in a growth cabinet at 25°C in 16 h photoperiod provided by cool-white light (Phillips; 30–40 $mmol/m^2$ s). Root induction was performed on full and half-strength MS medium supplemented with different concentrations of 2, 4-D (0.5, 1.0, and 2.0 µM). Shoot multiplication ratio was calculated by the final shoot number after 30 days of culture compared to initial number of shoots. *Ex-vitro* transplantation was carried out after 2 months by removing the rooted plantlets from the *in vitro* culture, rinsing them in water to remove the medium, followed by transfer to potting soil mixture under standard greenhouse conditions. The mean daytime and night time temperatures in the greenhouse were 25.2°C and 15.3°C, respectively. There was no supplemental lighting in the greenhouse and the average light level on the benches was 244 $\mu mol/m^2s$. The humidity of growth environment for the regenerated plantlets was maintained with an intermediate misting system with an initial frequency of 30 minutes intervals during daylight and 60 minutes intervals overnight. The misting frequency was reduced to half after 6 weeks in daylight and night, respectively. Sixty plants per triplicate were irrigated with half-strength MS solution every 24 h and all treatments consisted of ten replicates with the experiments repeated twice.

RESULTS

Nodal explants of *A. amygdalina* were incubated on full strength MS medium supplemented with BAP in combination with NAA. The callus development was observed in 2 week old cultures on all media tested (Table 1, Figure 1a) and it was found that more number of shoots developed on Nodal explants exposed to 10 µM NAA and 10 µM BAP with an average of 36 shoots per nodal explant than other treatments after 42 days (Figure 1b and Table 1). The treatment with BAP alone did not induce shoot organogenesis from node explants of *A. amygdalina*; however, addition of NAA to BAP containing medium also induced shoot organogenesis. Regenerated node explants were separated and sub-cultured on MS medium supplemented with various concentrations of BAP and Kinetin for further shoot proliferation and growth (Figure 1d) with half strength MS medium giving the best shoot proliferation results after 30 days of culture (Figure 2). Root initials were found to present after 2 weeks of culture with the highest rate of root development observed on half-strength MS medium after 42 days (Figure 1c; Table 2). The highest percentage for the rooting response was observed on half strength MS medium with about 7 ± 0.3 roots per regenerated node with an average length of 8.2 cm. However, the addition of 2, 4-D caused increased root formation, but with indirect rooting. Hence use of 2, 4-D medium proved helpful in root formation from callus. The rooted plantlets survived *ex-vitro* transplantation in the greenhouse normal conditions without any supplemental light. Plants fertilized with half strength MS solution every 24 h were able to grow and develop well formed leaves with characteristic morphology within 3 months (Figure 1e and 1f).

Table 1: Effect of different concentrations of BAP and NAA on shoot regeneration from Nodal explants after six weeks

Induction Media	Callusing	Regeneration	No. of Shoots/Node	Average size of Shoots	% age Response
MS + BAP (0.88 µM) + NAA (0.54 µM)	No response	-	-	-	-
MS + BAP (5 µM) + NAA (5 µM)	No response	-	-	-	-
MS + BAP (7.5 µM) + NAA (7.5 µM)	Moderate callusing	Direct regeneration	18 ± 0.1	4.4 cm	80 %
MS + BAP (7.5 µM) + NAA (10 µM)	Low callusing	Direct regeneration	21 ± 0.3	4.2 cm	70 %
MS + BAP (10 µM) + NAA (7.5 µM)	Low callusing	Direct regeneration	24 ± 0.4	4.7 cm	90 %
MS + BAP (10 µM) + NAA (10 µM)	Low callusing	Direct regeneration	36 ± 0.5	4.8 cm	90 %
MS + BAP (10 µM) + NAA (12.5 µM)	Moderate callusing	-	-	-	60 %
MS + BAP (12.5 µM) + NAA (10 µM)	High callusing	-	-	-	50 %
MS + BAP (12.5 µM) + NAA (12.5 µM)	High callusing	-	-	-	60 %
MS + BAP (15 µM) + NAA (15 µM)	No response	-	-	-	-

Table 2: Effect of plant growth regulators on rooting of *in vitro* raised shoots after six weeks

Rooting Media	No. of Roots		Mean size of Roots		% age Response
	Regenerated Shoot tip	Regenerated Node	Regenerated Shoot tip	Regenerated Node	
MS	3 ± 0.1	5 ± 0.2	6.3 cm	7.1 cm	90
Half strength MS	6 ± 0.3	7 ± 0.3	7.5 cm	8.2 cm	100
MS + 2, 4-D (1 µM)	9 ± 0.6	9 ± 0.2	3.7 cm	3.9 cm	80
MS + 2, 4-D (2 µM)	5 ± 0.2	6 ± 0.3	3.2 cm	3.4 cm	80

**Figure 1: *In vitro* plant regeneration system for *A. amygdalina* (a) Callus induced from nodal explants cultured for a period of 15 days.
(b) Multiple regenerated shoots grown on a medium supplemented with 10 µM NAA and 10 µM BAP.
(c) Root formation on the half-strength MS medium from the *in vitro* raised shoots.
(d) Proliferation and elongation of *in vitro* raised shoots on half strength MS medium.
(e) Whole plantlet transferred to soil pot for acclimatization.
(f) Young, healthy, mature plantlets in greenhouse after 3 months of growth**

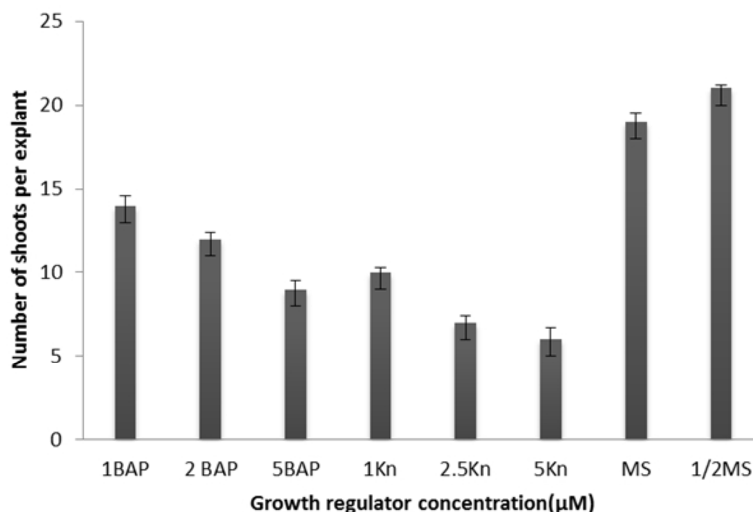

Figure 2: Effect of plant growth regulators on shoot multiplication from nodal explants (BAP = benzyl amino purine; Kn = Kinetin; MS = Murashige and Skoog medium)

DISCUSSION

Stem internode sections of *Artemisia scoparia* cultured on MS[9] basal medium added with different cytokinins and auxins showed prolific adventitious shoot and shoot bud formation on medium with high IAA (5.0 mg · 1^{-1}) and BAP (3.0 mg 1^{-1}). The stimulation of tissue growth to form adventitious roots and shoots depends on the ratio of auxin to cytokinin in the culture medium[10] and hence an efficient *in vitro* propagation protocol for *Artemisia amygdalina* D. was developed by the use of different plant growth regulators in different concentrations. The regenerative callus culture in the plant initiated by using different concentrations of MS medium supplemented with BAP (cytokinins) and NAA (auxins) is confirmed by various studies carried out on *Artemisia annua* L. using different concentrations of the same medium and the plant growth regulators[11,12]. The shoot proliferation of *A. amygdalina* on MS medium supplemented with auxins and cytokinins is also confirmed by a study on *Artemisia scoparia*[13] showing a prolific shoot formation on MS medium supplemented with IAA and BAP. The regenerative callus formation can be attributed to the fact that auxins are root promoting hormones and can work best in combination with cytokinins for shoot proliferation. The endogenous auxin/cytokinin level of explants can be made optimum for shoot proliferation and growth by exogenous supply of plant growth regulators. The micro shoots showed elongation and multiplication on MS + BAP medium. Isolated micro shoots showed rooting, elongation and multiplication on basal MS and MS (1/2) medium. MS medium fortified with 2, 4-D showed indirect rooting. Decreasing 2, 4-D concentration decreased the extent of callus formation. Thick, white and short roots were formed on this hormone supplemented medium. The best conditions of rooting were found to be both MS basal and MS (×1/2) with slight variation in the shoot number. These results are in concordance with the studies of Lualon where healthy regenerated shoots were elongated and rooted in MS medium without hormones[14]. In *Artemisia absinthium* direct rooting was achieved on full and half strength MS medium supplemented with different auxin concentrations[15]. MS basal was found to be best for rooting and elongation of isolated micro shoots as has been suggested by Sujatha and Kumari while working on *Artemisia vulgaris*[16].

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REFERENCES

1. Qaisar M. Asteraceae. Flora Pak 2006; 27: 120–121.
2. Rasool R, Ganai BA, Kamili AN Akbar S. Antioxidant potential in callus culture of *Artemisia amygdalina* Decne, Natural Product Research: Formerly Natural Product Letters; 2011. http://dx .doi.org/10.1080/14786419.2011.617749
3. Rather MA, Ganai BA, Kamili AN, Qayoom M, Akbar S, Masood A, Rasool R, Wani SH, Qurishi MA. Comparative GC–FID and GC–MS analysis of the mono and sesquiterpene secondary metabolites produced by the field grown and micropropagated plants of *Artemisia amygdalina* Decne. Acta Physiol Plant; 2011.
4. Dar AR, Dar GH, Reshi Z. Conservation of *Artemisia amygdalina*-A critically endangered endemic plant species of Kashmir Himalaya. Endangered Species Update 2006; 23: 34–39.
5. Rout GR, Samantaray S, Das P. *In vitro* manipulation and propagation of medicinal plants. Bio technol Adv 2000; 18: 91-120. http://dx.doi. org/10.1016/S0734-9750(99)00026-9
6. Nalawade SM, Sagare AP, Lee CY, Kao CL, Tsay HS. Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Bot Bull Acad Sin 2003; 44: 79-98.
7. Murch S, Peiris SE, Liu CZ, Saxena PK. *In vitro* conservation and propagation of medicinal plants. Biodiversity 2004; 5: 19–24. http://dx. doi.org/10.1080/14888386.2004.9712714
8. Pan ZG, Liu CZ, Saxena PK. Efficient plant regeneration from protoplasts of Egyptian medicinal plants *Artemisia judaica* L. and *Echinops spinosissimus* Turra. Plant Sci 2003; 165: 681–687. http://dx. doi.org/10.1016/S0168-9452(03)00220-6
9. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 1962; 15: 473–497. http://dx. doi.org/10.1111/j.1399-3054.1962.tb08052.x
10. Skoog F, Miller CO. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. In: Porter, HK (Ed.), the Biological Action of Growth Substances. Academic Press New York; 1957. p. 118–131.
11. Bartarya R, Srivastava A, Tonk S, Bhatnagar VP, Srivastava SS, Kumari KM. Larvicidal activity of *Artemisia annua* L. callus culture against *Anopheles stephensi* larvae. J Env Biol 2009; 30(3): 395-398.
12. Kamili AN, Bashir S, Shah AM. Regeneration of plantlets from petiole derived callus of *Artemisia annua*. J Res Dev 2003; 287-293.
13. Kothari SL, Chandra N. Adventitious Shoot Production from Stem Internode and Callus Cultures of *Artemisia scoparia* Waldst. et Kit. Journal of Plant Physiology 1986; 124(5): 409-412. http://dx. doi.org/10.1016/S0176-1617(86)80197-3
14. Lualon W, De Eknamkul W, Tanaka H, Shoyama Y, Putalun W. Multiple shoot regeneration and artemisinin production in *Artemisia annua* L. using thidiazuron. Z. Naturforsch. C-A. Journal of Biosciences 2008; 63(1-2): 96-100.

15. Zia M, Riaz UR Rehman, Chaudhary MF. Hormonal regulation for callogenesis and organogenesis of *Artemisia absinthium*. African journal of Biotechnology 2007; 6(16): 1874-1878.
16. Sujatha G, Kumari BDR. Effect of phytohormones on micro propagation of *Artemisia vulgaris* L. Acta physiol Plant 2007; 29: 189-195. http://dx.doi.org/10.1007/s11738-006-0023-0

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