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In vitro propagation of an important medicinal plant *Artemisia annua* L. from axillary bud explants

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ABSTRACT

An efficient in vitro micro propagation technique for multiple shoot induction has been developed for Artemisia annua L. using axillarybud explants in different concentrations and combination of plant growth regulators in order to produce multiple shoots. The results showed that the nodal segments with axillarybud cultured on MS basal medium fortified with 1.5mg/l BAP+0.5mg.l NAA facilitated favorable shoot induction. The well developed shoots were transferred on rooting medium (MS) supplemented with various concentration of IBA. The medium supplemented with 1.0mg/l of IBA, showed efficient root induction and further development of healthy roots. The well rooted plantlets were acclimatized and successfully established in field with 85% of survival.

Keywords: *Artemisia annua* L., *In vitro* propagation, Medicinal plant, Axillarybuds.

INTRODUCTION

Medicinal plants are the source of important therapeutic aid for alleviating human ailments [1]. *Artemisia annua* L. (sweet wormwood) is an important medicinal herb belongs to the family Asteraceae (Compositae), contains an anti malarial compound artemisinin, which is effective against *Plasmodium falciparum* [2]. *Artemisia annua* is an annual herb native to Asia, most probably China with a long history of use against hemorrhoids and malaria [3]. Artemisinin and its derivatives have also been reported to be an effective non- selective herbicide such as glyphosate [4]. It has also been reported to have anti cancer properties and have the ability to be selectively toxic to breast cancer cells [5].

In animal studies, artimisinin has been used in high oral doses in dogs and rabbits [6]. *Artemisia annua* L. leaves and crude extracts have been reported to be a good source of anti oxidant due to highest oxygen radical absorbance capacity (ORAC) [7,8]. This high ORAC is probably due to high content and diversity of its leaf flavanoids, including the newly reported C-glycosyl flavonoids as a possible component of the antioxidant and antiviral activity [9,10]. Biseriate glandular trichomes are the source of highly aromatic volatile oils, mainly artemisia ketone, 1-8-cineolecamphor, germacrene D, camphene hydrate, and α-pinene, β-caryophyllene, myrcene, and artemisia alcohol [11]. The essential oil of this plant was reported to contain 90% mosquito repellency against the mosquito *(Aedes aegypi)* that transmits yellow fever [12]. The production of artemisinin was also achieved by hairy root culture [13,14].

In vitro micro propagation and organogenesis of various Artemisia species have been previously established by using several explants in order to produce large number of plants, such as *A.vulgaris* [15], *A.annua* [16,17]. *In vitro* micro propagation technique provides many advantages over conventional propagation methods. Micro propagation through shoot tip culture, often utilized to maintain clonal fidelity would be a special advantage in this technique [18].

The present study is aimed to develop an appropriate and efficient micro propagation protocol from axillary bud explants of *A. annua* for the large scale production of plants. The protocol reported here could be used for the conservation it can also be used for genetic transformation study of this species.

MATERIALS AND METHODS

The healthy mother plants of *Artemisia annua* L. were collected from herbal nursery maintained by Forest department of Tamilnadu at Pollachi, Coimbatore and maintained in green house of Pachaiyappa's College, Chennai, Tamilnadu. The mother plants were authenticated by taxonomist in the Department of Botany, Pachaiyappa's College, Chennai. The explants such as shoot tips, auxiliary buds were collected from these plants for the present investigation. The explants were washed with running tap water to remove the dust particles available on the surface of the explants. Then the explants were immersed in soap solution for 30 min and washed thoroughly with tap water and brought it to aseptic condition. Then the explants were finally treated with 0.1% of $HgCl_2$ (w/v) for five minutes and rinsed thoroughly 3-4 times with sterile water to remove the traces of chemicals. Under the aseptic condition the nodal segment explants were resized to 1.00 to 1.50cm long and inoculated on to the culture medium.

Culture medium

All the media used in this study were based on Murashige and Skoog (MS) basal medium [19] supplemented with 3% of sucrose and fortified with different concentration and combination of growth regulators such as 6-benzyl amino purine (BAP) 0.5-2.0 mg/l, α-Napthalene acetic acid (NAA) 0.1-0.5 mg/l , Indole 3-butyric acid (IBA) 0.1-1.00mg/l and Indole 3-acetic acid (IAA) 0.1-1.00mg/l to study their response on multiplication of shoots and rooting. The p^H of the media was adjusted to 5.8.

Initiation

The axillary buds of *Artemisia annua L.* were inoculated on the culture medium under the aseptic condition. All these cultures were incubated under the temperature of 25 ± 2^0C and the light intensity of 2000-4000 Lux. The cultures were maintained with the photoperiod regime of 16 hr light and 8 hr dark. The multiple shoots formed in *in vitro* were isolated and sub cultured on fresh medium for further multiplication and rooting. The number of shoots, roots, length of shoots and roots in 30 days of culture were recorded based on the periodical observation.

The cultures were observed on daily basis and number of shoots, roots and length of shoots, roots were recorded. The collected data were analyzed by one way ANOVA followed by Tuky's HSD test values represent the mean ±SD of eight replicates and all experiments were repeated three times, mean difference is significant at the 0.05 level.

The well developed shoots with roots were washed thoroughly with running tap water to remove the traces of agar. The healthy plants were transferred to protrays containing the primary hardening mixtures of sand and vermiculite in the ratio of 1:1 to achieve maximum survival rate.

RESULTS

The axillary buds of *A. annua* cultured on the MS basal medium fortified with different concentrations of BAP (0.5-2.5mg/l) alone and in combination with NAA (0.1-0.5mg/l) **(Table-1)**. Multiplication of shoots occurred in all concentration and combinations of growth regulators, but the number and the length of shoots per explants varied. The explants were cultured on MS medium without growth regulators (control) showed less shoot induction with mean number of shoots 01.50±0.53 with mean shoot length (2.06±0.49). Among the various media combinations, MS medium fortified with BAP-1.5mg/l + NAA-0.5mg/l is responded very effectively for the successful induction and the multiplication of shoots, with the maximum of 27.75±1.28 shoots per explants and with mean length of 6.56±0.42 **(Table-1)(Fig: a,b)**. In an another set of experiment, the MS medium supplemented with BAP- 0.5, 1.0 mg/l alone and in combination with NAA-0.5mg/l, showed poor induction of shoots per explant **(Table-1)**. MS medium fortified with BAP- 2.0 mg/l individually and in combination with NAA-0.5mg/l produced fur like shoots, which are not be suitable for next phase of multiplication. Hence, these combinations were not appropriate towards the production of quality shoots.

For root induction, individual shoots were placed on the medium supplemented with different concentrations of IBA and IAA **(Table-2)**. Root induction was noticed in all the concentrations, but with varied response with reference to the number and length of the roots per shoot. Among the various concentrations, favorable root induction was noticed on MS+IBA-1.0mg/l in terms of average number of roots (10.75±1.48) with mean root length of 5.93±0.77 cm per shoot **(Table-2)**. In the case of IAA, out of five different concentrations of IAA, the optimal root induction was observed on MS with 1.0 mg/l of IAA. This media showed the maximum average number of roots (6.50±0.92)

with mean length of 2.20±0.42 cm per shoot **(Table-2)**. After 30 days, *in vitro* developed shoots were harvested and washed with running tap water and transferred to green house for primary hardening process **(Fig:1c&d)**. After 50 days, the primary hardened plants were transferred to polybag for secondary hardening and 95% of the survival rate was noticed in the end of the secondary hardening phase.

Table-1: Effect of growth regulators on shoot induction and multiplication

Growth regulators		Number of shoots	Length of shoots(cm)
BAP (mg/l)	NAA (mg/l)	Mean±SD	Mean±SD
0.00	0.00	01.50±0.53	2.06±0.49
0.50	0.00	05.12±0.83	2.87±0.35
1.00	0.00	16.50±1.60	3.68±0.37
1.00	0.50	17.62±0.92	3.75±0.46
1.50	0.50	27.75±1.28	6.56±0.42
2.00	0.00	19.12±1.35	4.44±0.42
2.00	0.50	16.37±1.18	4.81±0.70

The values represent the mean±SD of eight replicate s and all experiments were repeated thrice, mean difference is significant at the 0.05 level.

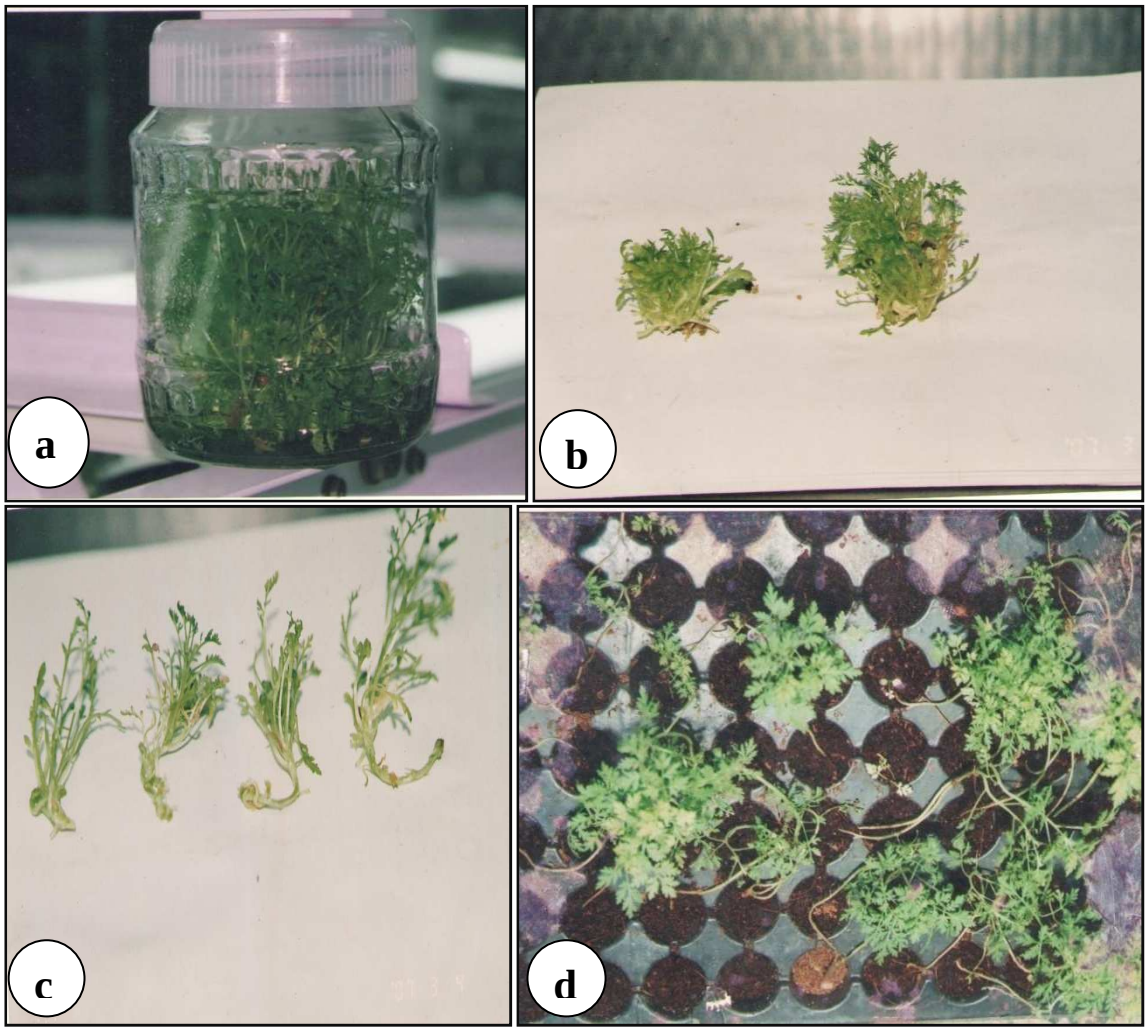

Figure:1 a-Multiple shoots on MS+BAP(1.5mg/l) + NAA (0.5mg/l), b-Clump of multiple shoots removed from bottle, c-Washed healthy shoots for hardening, d-healthy shoots under hardening on plastic tray containing hardening mixture

Table-2: Effect of various auxins on root induction

Growth regulators		Number of roots/shoot	Length of roots (cm)
IBA (mg/l)	IAA (mg/l)	Mean±SD	Mean±SD
0.10	0.00	06.37±0.91	4.28±0.55
0.30	0.00	07.75±1.28	3.56±0.41
0.50	0.00	08.37±1.18	5.43±1.01
0.70	0.00	07.50±0.92	3.68±0.46
1.00	0.00	10.75±1.48	5.93±0.77
0.00	0.10	05.12±0.64	3.37±0.51
0.00	0.30	06.37±1.06	3.07±0.38
0.00	0.50	08.12±1.24	5.91±0.96
0.00	0.70	05.87±0.83	2.70±0.53
0.00	1.00	06.50±0.92	2.20±0.42

The values represent the mean±SD of eight replicates and all experiments were repeated thrice, mean difference is significant at the 0.05 level.

DISCUSSION

Morphogenic responses vary with different concentration and combination of plant growth regulators [20]. *In vitro* propagation of plants through tissue culture technique has been achieved by using the appropriate concentration and combinations of plant growth hormones [21,22]. *In vitro* micro propagation is an advanced technique which offers a large number of genetically uniform and disease free plants in limited duration and space [23]. The moderate concentration of cytokinin (BAP) is highly sufficient towards the successful production of quality shoots. The present findings are coincident with the earlier report by Ganesan *et al.*, [24]. The present result showed that, when the concentration of BAP is increased gradually the multiplication rate is reduced to a greater extent, which is also akin to the previous report of Ganesan *et al.*, in *Asrtemisia annua* L.[24] The medium fortified with BAP (1.0 & 2.0 mg/l) alone and in combination with NAA (0.5mg/l) were showed nearly similar results. Regarding the rooting, the media enriched with MS+IBA 1mg/l showed optimum rooting and this is also in line with the previous reports in *Artemisia vulgaris* L by Sujatha Govindaraj *et al.*, [15]. IAA was used as a potential auxin for root induction in various crops like *Arachis stenosperma* and *A. villosa* [25]. Increasing the concentration of IAA to 0.5mg/l and above gradually leads to the reduction of root induction. A similar result was also observed in *Artemisia vulgaris*L [15].

CONCLUSION

The *in vitro* propagation techniques help in multiplication of plant species, which have restrictions of conventional propagation. The present study has provided an efficient protocol for plant regeneration through axillarybud culture in *Artemisa annua L.* This would help to produce the large scale production of disease free planting material.

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