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AN EFFICIENT MICROPROPAGATION PROTOCOL OF AN ELITE CLONE EC-353508 OF ARTEMISIA ANNUA L., AN IMPORTANT ANTIMALARIAL PLANT

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ABSTRACT

An efficient *in vitro* propagation system for an elite clone EC-353508 of *Artemisia annua* L., an important antimalarial drug plant has been developed. The best organogenic response, including adventitious multiple shoots and their elongation, was obtained when nodal explants were cultured on MS medium supplemented with 10µM 2-isopentenyl adenine (2iP). An average of 17.7±0.88 shoots with an average shoot length of 5.25±0.17 (cm) was differentiated per explant. Excised *in vitro* shoots organized roots in cent percent cultures when subcultured on half MS basal medium augmented with 5 µM α-naphthalene acetic acid (NAA). The plantlets were gradually hardened and transferred to sterile garden soil: soilrite (1:1) mixture for acclimatization. This is the first report of an efficient and cost effective protocol for direct shoot multiplication from nodal explants in an elite clone of *Artemisia annua*.

KEYWORDS

Artemisia annua L., Adventitious shoots, 2iP (2-isopentenyl adenine), NAA (naphthalene acetic acid), Soilrite, Acclimatization

INTRODUCTION

Artemisia annua L. a native of China, belongs to the family Asteraceae and is a tall aromatic annual herb. It is well known for artemisinin, a unique bioactive compound which is used against cerebral malaria caused by the endoparasite *Plasmodium falciparum*[1]. Malaria is a global health problem that threatens half of the world's population and an estimated 243 million cases led to nearly 8,63,000 deaths in 2008[2]. The development of resistance to chloroquine and the appearance of multidrug resistant parasites impair all possible treatment of malaria. Therefore, search for new anti-malarial compounds from plants is of increasing clinical interest. One hope for an effective antimalarial agent to encounter increasing problems of resistance to the currently applied drugs lies within artemisinin and its semi-synthetic derivatives. In 2004, the World Health Organization (WHO) recommended that artemisinin-based combination therapy (ACT) should be the norm for the treatment of falciparum malaria in most endemic countries came into effect[3]. More recently, they have also shown potent and broad anticancer properties in cell lines and animal models[4].

Artemisinin is a sesquiterpene lactone containing an endoperoxide bridge that is considered essential to its antimalarial action: haem-catalyzed cleavage of the peroxide generates unstable free radicals to which malaria parasites are particularly sensitive[5]. Artemisinin, isolated from the aerial parts of the plant is highly effective against multi-drug resistant *Plasmodium* spp., but is in short supply and unaffordable to most malaria sufferers[6]. The contents of the artemisinin in *Artemisia annua* plants are relatively low, ranging from 0.01 to 0.5% on a dry weight basis (DW), depending on the *Artemisia annua* variety. This makes artemisinin a relatively expensive drug (cost of 1 Kg ranges between US $350-$1700)[7] while the demand for large quantities of cheap artemisinin is growing proportionally with the increased resistance to the commonly used anti-malarial drugs. Artemisinin has rapid action and is effective against chloroquine-resistant malarial parasites and has a different mode of action from existing blood schizonticides. Artemisinin has also been found to be active for treating skin diseases[8] and as a natural herbicide[9]. Synthesis of artemisinin has not proved to be commercially feasible[10,11] and it is obtained with low yield from natural sources[12,13,14,15]. The highest artemisinin content has been found in leaves and flowering tops of the plants which in turn was influenced by several factors such as plant variety, cultivation conditions and geographic localization[14,16]. Incidentally, the yield is insufficient to meet the demands of the industry (1000 kg dry leaves yielding only 1 kg artemisinin). Several *in vivo/in vitro* studies had been earlier reported[17,18,19] to increase the biosynthesis of artemisinin and related compounds however, no significant enhancement has been recorded till date. This limits *Artemisia annua* plants as the sole source of artemisinin for which a large amount of the plant material is required to extract appreciable quantities of the drug. This subsequently leads to depletion of the natural resources and puts the plants at risk of being endangered. Thus, *in vitro* regeneration techniques, which offer powerful tools for germplasm conservation and the mass multiplication of many threatened plant species,

have gained increasing importance as a promising alternative[20]. *In vitro* propagation is still seen as an important tool in conservation as it could provide complimentary conservation option for plant species with limited reproductive capacities[21]. Nevertheless, a tissue culture stage is a prerequisite for most current transformation protocols for the ultimate recovery of transgenic plants[22]. This was one of the motivating factors behind this particular study, as a tissue culture system for the genetic transformation of *A. annua* is currently required to further enhance the production of secondary metabolites.

In view of the aforesaid problems the current investigations were undertaken using elite artemisinin yielding *Artemisia annua* clone for development of a simple, cost effective and commercially viable micropropagation protocol for generation of a large number of clonally propagated plantlets and their transfer into the field. Though, some fragmentary reports[17,18,19] are available on tissue culture of *Artemisia annua*, however they do not prove to be cost effective for implementation on a large scale. We hereby, report a simple, cost effective and repeatable protocol for large scale propagation of an elite clone of *Artemisia annua,* thereby providing immense scope for artemisinin production at commercial level.

MATERIAL AND METHODS

(i) Plant material:

Plants were grown *in vitro* on MS basal medium from the seeds of an elite clone of *Artemisia annua* L. (Asteraceae) procured from NBPGR (Delhi). Seeds were surface sterilized with 1% teepol for 20 minutes followed by 1% bavistin for 20 minutes and 0.1% mercuric chloride ($HgCl_2$). Before inoculation, the seeds were rinsed thrice with sterile distilled water. The sterilized seeds were inoculated for germination in MS[23] basal medium. For multiple shoot differentiation, 2 cm long nodal explants excised from 30 days old seedlings were cultured on MS medium supplemented with various growth regulators (Table 1).

(ii) Culture conditions:

MS medium was gelled with 0.7% agar and the pH adjusted to 5.8 prior to autoclaving for 20 minutes at 121^0 C and 1.06 kg cm^{-2}. Cultures were maintained in room having $25±2^0$ C temperature, 1045 lux (40W cool fluorescent tubes and 60 W incandescent lamps) light and 55±5% relative humidity. All subsequent subcultures were carried out at two weeks interval.

(iii) Multiple shoot induction and plantlet regeneration:

The nodal explants (1-2 cm) excised from one month old *in vitro* grown seedlings were inoculated vertically on MS medium supplemented with different concentrations of BA (6-benzyl adenine) (1, 5, 7.5 and 10µM), KN (kinetin) (1,5,7.5 and 10µM) and 2iP (2-isopentenyl adenine) (1, 5, 7.5 and 10µM) individually for multiple shoot induction. For each treatment, a total of twelve replicates each with one explant was inoculated; therefore, twelve explants per treatment were tested. The multiple shoots initiated were subcultured at every two weeks for 60 days. A control group was maintained (basal medium without hormones) to record the frequency of response.

(iv) Rooting:

To induce rooting, individual elongated shoots (2.3 – 6.0 cm long) were isolated individually and transferred to MS medium containing different concentrations of IBA (indole butyric acid) (0.1, 1, 5 and 10µM) and NAA (naphthalene acetic acid) (0.1, 1, 5 and 10µM). One culture set was inoculated on half basal MS medium without the addition of auxins and kept as control.

(v) Acclimatization and field transfer:

Plantlets with well developed roots were removed from the culture medium and after

washing the roots gently under running tap water to remove the adhering medium, the roots were washed with 1% bavistin solution. Further, plantlets were transferred to thermocol cups, containing autoclaved garden soil and soilrite (1:1). Each of the cups was irrigated with MS liquid medium every two days for three weeks followed by tap water for two weeks. The potted plantlets were initially maintained under culture room conditions (five weeks) and then transferred to normal laboratory conditions (four weeks). After 60 to 70 days, the plantlets were transferred to the greenhouse. The plants were irrigated with tap water every 24 h under greenhouse conditions. The survival frequency and growth characteristics were examined.

(vi) Statistical analysis:

The design used for all experiments were a complete randomized block and each experiment consisted of one explant per culture tube and twelve replicate culture tubes per plant growth regulator treatment. All of the experiments were repeated twice, and the data were analyzed using the Duncan's multiple range test (at p = 0.05).

RESULTS AND DISCUSSIONS

(i) Seed germination and explants establishment:

The important part of the present study was the preparation of a contamination free explant source. This was achieved by using *in vitro* germinated seedlings as explants. The size of the seeds varied from 0.5 – 0.8mm. Seed germination was observed after two weeks of incubation (Fig. 1a). Nodal explants (1 – 2 cm) excised from *in vitro* four week old seedlings were used in the present study for enhanced multiple shoot production.

(ii) Effect of cytokinins (ba, kn and 2ip) on shoot regeneration:

Nodal explants (1-2 cm in length) excised from the four week old seedlings were cultured on MS basal augmented with different concentrations (1, 5, 7.5 and 10μM) of cytokinins; BA, Kinetin and 2iP (Fig. 1b). The best response was obtained on MS+10μM 2iP where an average of 17.7±0.88 shoots with an average shoot length 5.3±0.17 cm was directly differentiated in 100% of the cultures (Fig. 1c). Kinetin proved to be the least responsive amongst all the cytokinins employed. Any efficient micropropagation protocol advocates the necessity of maximum biomass production with minimum expenditure of nutrients and growth regulators. The earlier reports[17,18,19]on micropropagation of *Artemisia annua* are not only fragmentary but also cumbersome which seriously hinders the proficiency of any regeneration protocol employed on a larger scale.

The present investigation highlights the development of a simple, cost effective and commercially feasible protocol which is not only viable in nature but also leads to generation of a large number of clonally propagated elite plantlets within a short duration. In the given work, seedling derived nodal explants were employed for direct induction of multiple shoots on MS medium supplemented with a single cytokinin (BA, kinetin or 2iP) (Table-1). In contrast, Gulati et al. (1998)[19] used a very complex combination of auxin, cytokinin, gibberrelic acid and amino acids (NAA+BA+GA_3+L-asparagine+glutamine+L-arginine+cysteine hydrochloride) supplemented to MS medium for indirect regeneration of multiple shoots through different explants (leaf, stem, inflourescence, hypocotyl). In other earlier reports[17,18] multiple shoots were obtained via callus on MS medium augmented with high concentrations of NAA+BA. However, no detailed record of the number of multiple shoots, shoot length, rhizogenesis and hardening of the plants has been mentioned. Cytokinins have the property of breaking bud dormancy[24,25,26]. The MS+10μM 2iP proved best for eliciting average

number of 17.7±0.88 shoots within four weeks. Our results in this elite clone are in contrary to all other earlier reports where BA instead of 2iP gave significant results. The positive role of 2iP in multiple shoot formation has been reported earlier in ornamental cultivars of Ericaceae[27], *Ixora coccinea*[28], *Gardenia jasminoides* Ellis[29], *Vaccinium cylindraceum* Smith[30] and *Aristolochia indica*[31]. Cytokinins play an important role in various processes in the growth and development of plants, including the promotion of cell division, the counteraction of senescence, the regulation of apical dominance and the transmission of nutritional signals[32].

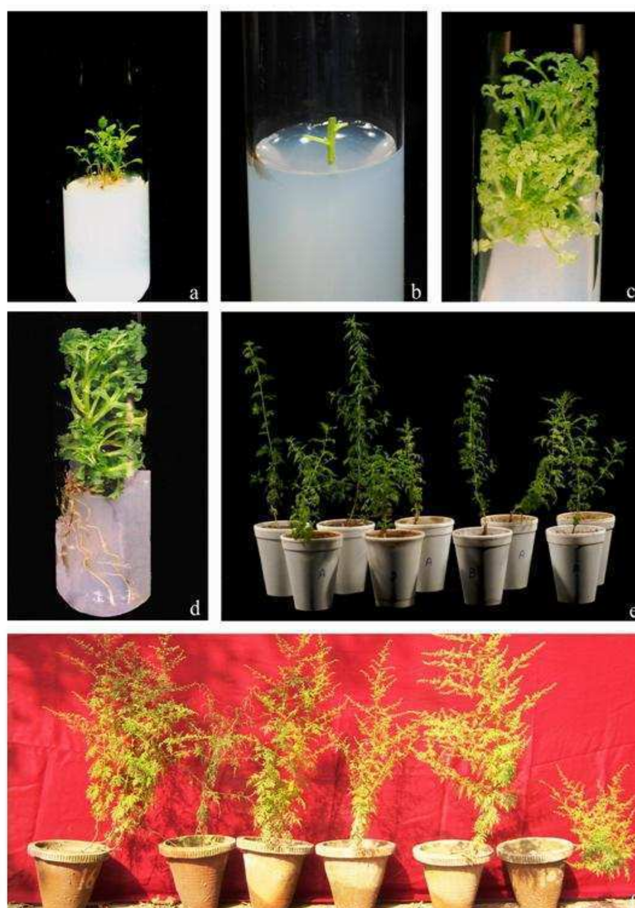

Fig. 1a – f.

Morphogenic response of Artemisia annua L. elite clone EC-353508. (a) Germination of seeds of Artemisia annua L. on MS basal medium after 2 weeks of inoculation; (b) An excised aseptic nodal explant on MS basal medium adjuvanted with 10 µM 2iP; (c) Multiple shoots regeneration on MS basal medium augmented with 10 µM 2iP; (d) Induction of roots on in vitro regenerated shoots on ½ MS basal augmented with 5 µM NAA after 4-weeks of culture; (e) Acclimatization of in vitro derived plantlets grown on soilrite : sterile garden soil (1:1) after 8 weeks; (f) Tissue culture raised plants transferred to field after 20 weeks.

Previous experiments have revealed that 2iP, a free base cytokinin could bind to the cytokinin receptor in plants and therefore, is grouped in the active forms of cytokinins[33].

The primary cytokinin signal transduction pathway identified is similar to bacterial two-component phosphorelay paradigm. In a two-component system, a histidine kinase detects

the extracellular stimulus and becomes phosphorylated at the conserved histidine residue. The phosphoryl group is then transferred to the receiver domain of a response regulator that regulates the activity of its output domain. The cytokinin signaling events has been defined from the perception of cytokinins at the cell surface to changes in gene expression in the nucleus. However, additional pathways may exist for the perception of cytokinin and the transduction of its signal. Two important additional potential outputs are the regulation of protein stability and calcium signaling[34].

Table 1

Differential morphogenic responses of nodal explants of elite clone, EC-353508 of Artemisia annua L. plant on MS medium supplemented with different cytokinins, after one month of inoculation.

Growth Regulator (µM)			*Percentage Responding cultures	*Average No. of shoots	*Average Shoot length (cm)
BA	KN	2iP			
0			0	0	0
1			100^{e}	5.4±0.45^{a}	4.4±0.10^{b}
5			100^{e}	8.8±0.55^{b}	5.0±0.15^{c}
7.5			100^{e}	10.8±0.40^{d}	4.8±0.12^{b}
10			100^{e}	16.3±0.39^{e}	6.0±0.12^{d}
	1		66.7^{c}	5.9±0.91^{a}	2.4±0.36^{a}
	5		100^{e}	13.7±0.96^{e}	4.0±0.12^{b}
	7.5		58.3^{b}	6.2±0.15^{a}	2.8±0.50^{a}
	10		50^{a}	8.4±0.94^{b}	2.3±0.49^{a}
		1	75^{d}	8.9±0.08^{c}	3.0±0.43^{a}
		5	100^{e}	10.8±0.70^{d}	4.6±0.16^{b}
7.5			100^{e}	15.1±0.82^{e}	4.4±0.17^{c}
		10	100^{e}	17.7±0.88^{f}	5.3±0.17^{d}

**The values represent the means (±SE) of two independent experiments. A total of 12 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (P=0.05).*

(iii) Rooting of shoots:

Individual shoots were excised from the micropropagated shoots and transferred immediately to rooting medium augmented with 0.1, 1, 5 and 10µM of IBA or NAA. The rooting frequency was found to be optimal on media supplemented with 5µM NAA amongst all the concentrations tried (Fig. 1d). Roots were visible within two weeks following the transfer of elongated shoots to the rooting medium. A 100% rooting response was noted, a much poorer response for the control group. The number of roots ranged from 1.4 ± 0.37 to 10.7 ± 0.55 and the maximum root length observed was 6.2 ± 0.18 cm. Similar to our findings, NAA was reported as a potential auxin for rooting in *Pinellia ternate*[35], *Robinia pseudoacacia*[36] *Citrus aurantifolia* and *Citrus sinensis*[37], *Clitoria ternate*[38]. NAA at concentrations above 5µM gradually led to a decrease in the frequency of root regeneration (Table 2).

Table 2
Effect of different auxins on rooting of in vitro derived shoots of Artemisia annua L. cultured on MS medium. Data were recorded after 30 days of inoculation.

Growth Regulator(μM)		*Percentage responding cultures	*Average No. of roots	*Average root length (cm)
IBA	NAA			
0	0	50[a]	1.4±0.37[a]	2.8±0.72[a]
0.1		66.7[b]	1.6±0.25[a]	3.7±0.56[b]
1.0		83.3[c]	3.4±0.54[b]	3.9±0.43[b]
5.0		100[d]	7.0±0.29[d]	5.1±0.16[c]
10		83.3[c]	6.5±0.69[d]	5.0±0.49[c]
	0.1	66.7[b]	2.3±0.46[b]	3.9±0.62[b]
	1.0	100[d]	4.0±0.35[c]	6.2±0.18[d]
	5.0	100[d]	10.7±0.55[e]	3.6±0.18[b]
	10	66.7[b]	3.5±0.66[c]	1.8±0.28[a]

**The values represent the means (±SE) of two independent experiments. Total 12 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (P=0.05)*

The hormone mediated root induction and subsequent growth is dependent on certain factors which may trigger the complete chain of events that influence the ability of cultured cells to grow in an organized fashion. Plant tissues, therefore, must have receptors for hormones. These hormones interact with specific receptors that reside either on cell membrane or within the cytoplasm[39]. Affinity and concentration of receptors on the surface of the target tissues determine the type of response. Specific binding site for auxin have been identified[40]. A class of proteins called expansins mediates the proton ability to cause cell wall loosening[41]. These expansins break the hydrogen bonds between the polysaccharide components of the wall[42]. Proton (H^+) pumping and lowering of cytosolic pH result in an elevation of intracellular calcium level[43]. Both cytosolic pH and calcium ions act as second messengers in early auxin action[44]. Calcium ions, either themselves and or along with calcium binding proteins e.g., calmodulin activate the protein kinase cascade which in turn activates other proteins, including the transcription factors[45]. These factors presumably interact with the auxin-response elements and regulate the expression of auxin-inducible or auxin-responsive genes and exert its effect on cell cycle and stimulate cell division[46].

(iv) Acclimatization and field transfer:
After eight weeks, plantlets from the rooting medium were transferred into thermocol cups filled with soilrite and sterilized soil (1:1) for hardening (Fig. 1e). Plantlets were initially maintained under culture room (25 ± 1^0C) conditions for five weeks and then transferred to normal laboratory conditions for about four weeks. The plantlets were finally transferred to greenhouse conditions. Hundred percent plantlet survival was seen after hardening on garden soil and soilrite (1:1) for eight weeks. The initial growth in plant height was 18.5 cm to 25 cm during first five weeks of acclimatization and plants were transferred to the field (Fig. 1f). Further, after 8 – 20 weeks

of transfer, substantial increase in plant height was observed. In all the individual mature plants, stems were slender and the presence of minute hairs on the stem and lower leaf surface was observed. Flowering was observed after a period of 20 weeks and there was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics. In addition, all the tissue culture raised mature plants were free from any external defects.

Thus, this protocol is superior over the others as it paves way for the direct regeneration in an elite clone of *A. annua* in a mass scale although indirect regeneration through callus has been reported by earlier workers[17,18,19].

In conclusion, the present communication reports for the first time a cost effective, replicable and complete micropropagation protocol in an elite clone (EC 353508) of *A. annua.* This protocol appears to be highly effective and could be used as a means for large scale propagation of superior genotypes of *A. annua*- a medicinal plant possessing significant antimalarial activity. Further *in vitro* trials are in progress for improvement of artemisinin in *A. annua* employing genetic engineering techniques.

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