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# *In Vitro* Direct Organogenesis and Micropropagation of *Artemisia annua*

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## ABSTRACT

***Artemisia annua* is considered one of the important *Artemisia* species that produces aromatic oils and important medical compounds, such as Artemisinin known to be very effective against *Plasmodium falciparum*, responsible for cerebral malaria. The present investigations were initiated to develop a protocol for the effective direct organogenesis and propagation of *Artemisia annua*. Leaf, petiole, internodes and cotyledon explants were used for organogenesis. High percentages of direct regeneration were obtained from different kind of explants on medium supplemented of TDZ (Tidiazuron), with high number of bud formation. The best concentration of TDZ was 1mg/L. The morphogenetic capacity of explants taken from seedlings was higher than explants taken from plants cultured in greenhouse. Our investigation, led to the development of a mass propagation protocol with good multiplication rate, and good rooting percentage of buds formed from different kind of explants. It is concluded that our findings are very important for the gene transformation and for the regeneration of true to type plants in *A.annua*.**

**Key words:** *Artemisia annua*, Microropagation, TDZ, Direct organogenesis

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# التعضي والإكثار الخضري الدقيق لنبات *Artemisia annua.L* بزراعة النسج

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## الملخص

**يعد نوع النبات آرتيميزيا آنا *Artemisia annua* من الأنواع النباتية الطبية المهمة لاحتوائه على زيوت عطرية ومواد طبية مهمة مثل الآرتيميزينين شديد الفعالية ضد بكتريا البلاسميديوم التي تسبب مرض الملاريا. في هذا البحث، تم الحصول على تكوين البراعم مباشرة دون المرور بمرحلة الكالوس على أجزاء نباتية مختلفة؛ أوراق، وأعناق الأوراق، وسلاميات، وفلقات. وقد تم الحصول على أعلى نسبة من البراعم المتكونة في العينات النباتية المزروعة في وسط مغذ يحوي الهرمون TDZ تديازورون بتركيز 1 مغ/ل. تختلف استجابة الأجزاء النباتية للتشكل البرعمي بحسب نوعها ومصدرها، فقد دلت التجارب المنفذة ان العينات النباتية المأخوذة من النباتات البذرية المنبتة داخل الأنابيب قد أعطت استجابة للتبرعم أكثر من العينات النباتية المأخوذة من نباتات مزروعة في البيت الزجاجي. في هذا البحث تم تطوير تقنية للتكاثر الخضري الدقيق ولتشكل البراعم المباشر تفيد بالحصول على نباتات مشابهة في تركيبها الوراثي للنباتات الأم، كما يمكن إكثار السلالات المنتخبة التي تنتج نسبة عالية من مادة الآرتيميزين، كما تفيد التقنية الموضوعة في تطبيق تقنية التعديل الوراثي حيث تم الحصول على معدل إكثار وتجذير جيدين من مختلف الأجزاء النباتية المزروعة.**

**الكلمات المفتاحية:** آرتيميزيا آنا، تديازورون، التشكل البرعمي، التكاثر الخضري الدقيق.

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## Introduction

Medicinal plants are source of important therapeutic aid for alleviating human ailments (Dev. 1997). All *Artemisia* species produce aromatic oils and important compounds, such as artemisinin produced by *Artemisia annua*, very effective against both *Plasmodium vivax* and *Plasmodium falciparum*, the latter being responsible for cerebral malaria (Klayman 1985; Sy and Brown 2001).

*Artemisia scoparia* is known for antiseptic and strong antibacterial activity against different gram positive and negative bacteria (Yeung and Che, 1985). Scoparone extracted from *A. Scoparia* is known to reduce cholesterol and triglyceride level in blood (Lee *et al*., 2003).

Artemisinin is a rare sesquiterpene lactones end peroxide isolated from *A.annua* (*Asterceae*), it was recommended as a medicine against malaria.

To date, plantation of *A. annua* are the main source of commercial artemisinin. However, the artemisinin content in plants is highly variable ranging from 0.01- 0.42% (mg/g, dry weight) (Ferreira *et al*., 1997). Unsuccessful attempts have been made to produce artemisinin through callus culture (He *et al.,* 1983) or shoot culture (Fuzele *et al*., 1991). The production of artemisinin was reported by hairy root culture (Weathers *et al.*,1994; Xie *et al.,* 2000), culturing leaf blade and petiole segments of *A.annua in vitro*, explants were infected with *Agrobacterium rhizogenes*, promoted hairy root induction. Several clones of hairy roots were selected for their artemisinin production (Xie *et al.*, 2000; and 2001).

Micropropagation and organogenesis of different Artemisia species have been previously established by using several parts of plants, in order to obtain large number of plants, such as *A.scorpia* (Aslam *et al*., 2006); *A.vulgaris* (Govindaraj *et al.*, 2008); *A. mutellina* (Mazzetti and Donato, 1998). An efficient *in vitro* method for multiple bud induction and regeneration has been developed in *Artemisia annua*, using stem explants (Lualon *et al.,* 2008); or by using young inflorescence segments.

*In vitro* direct organogenesis of different parts of *Artemisia annua* was investigated, in this research, to obtain a large number of plants true to type. The ultimate goal was the multiplication of the selected clones with high levels of secondary metabolites and the utilization of the protocol in any future genetic transformation of this species.

## Material and Methods

Experimental studies were conducted on *Artemisia annua* in Plant Biology Department at North Carolina State University, USA at 2008-2009.

**Plant Material:**

*Artemisia annua* selected seeds were collected and planted in pots, in a phytotron, under short light conditions at 24°C. Plant aged of three months were used as a source of explants (Fig.1). Shoots length of 5-10 cm were isolated and surface disinfected by dipping in 70% ethanol for one minute, then immersing for 15 min. in 20% Clorox (a commercial preparation of sodium hypochlorite, with 5.25% active chloride), followed by three washings in sterile distilled water.

**Fig. 1. Mother plant of *A. annua***

Seeds of *Artemisia annua* were sterilized by dipping in 70% ethanol for one min, followed by three washing in sterile distilled water, then cultured in MS medium 1 (table 1), to obtain seedlings necessary for experimental studies.

**Culture Inoculation**:

Shoot apex, internodes, nodes, leaves and petioles from mother plants, and Cotyledons, petioles, and leaves from seedlings were isolated and inoculated into test tubes (25x150 mm) containing 12 ml of medium 2 and 3 (table 1), and incubated for one week in the dark under ambient growth room conditions 24±1°C, 50-60% relative humidity. One week later, explants (7-15 mm length) were transferred to 16h photoperiod under white light (40 µmol m² S) to study their genetic capacity on bud organogenesis.

Two cytokinins, Benzyl Amino Purine (BAP) and Thidiazuron

(TDZ), were used at different concentrations (0-0.2-0.6 and 1mg/l). 24 explants from each kind of explants were used per treatment. Experiments were repeated twice. All media were adjusted to pH 5.7 and then sterilized by autoclaving at 115° C for 30 mins.

**Shoot multiplication**

Buds formed in the first stage were divided and transferred to multiplication medium 4 (Table 1). The medium containing 0.5 mg/l of BAP was used for multiplication stage. Multiple shoots initiated were subcultured on the same medium one each 30 days. The shoots were maintained in the same conditions as previously mentioned.

**Rooting and transplantation**

Individual shoots were isolated and transferred to rooting medium5 (table 1). Indole butyric acid (IBA) was used at a concentration of 1mg/l, to study its effect on root formation of *A.annua.*

Transplantation *in vivo* plantlet with well developed roots was washed gently under running tap water to remove adhering medium. Rooted shoots were transferred to plastic containers (45x30x14cm) holding sterilized peat moss. The plants were irrigated in distilled water every two days for two weeks, followed by tap water for two weeks. The transferred plants were maintained in a greenhouse to develop normally.

**Data analysis:**

Several parameters were recorded, infected percentage, survival (%), bud regeneration (%), shoot multiplication rate (mean), rooting percentage, number of roots formed, and Percentage of survival plants developed in the greenhouse. The initiation and development of bud during the culture were observed under microscope, from the beginning of bud initiation under the bud formation.

Data were submitted to the variance analysis using ANOVA procedure. Duncan's test was employed to calculate significant differences at P= 0.05.

**Table 1. Media used for *Artemisia annua* regeneration**

| Media composition | Medium I | medium 2 | Medium 3 | Medium 4 | Medium 5 |
|---|---|---|---|---|---|
| MS mineral salts | MS | ½ MS | ½ MS | MS | ½ MS |
| Thiamin (mg/l) | 1 | 1 | 1 | 1 | 1 |
| Inositol (mg/l) | 100 | 100 | 100 | 100 | 100 |
| IBA (mg/l) | 0 | 0.1 | 0.1 | 0.1 | 1 |
| BAP (mg/l) | 0 | 0.2 - 1 | 0 | 0.2 | 0 |
| TDZ (mg/l) | 0 | 0 | 0.2 -1 | 0 | 0 |
| Sucrose (g/l) | 30 | 20 | 20 | 30 | 30 |
| Agar g/l | 3 | 3 | 3 | 3 | 3 |

I= Germination medium, 2 and 3= Bud organogenesis media, 4 = Multiplication medium, 5= Rooting medium.

## Results and Discussion

Surface sterilized seeds were germinated on MS (Murashige and Skoog, 1962) medium (Table 1). The germination percentage was more than 80% after one week of culture at 24° C (Fig. 2).

**Fig. 2. seedling plant of *A.annua***

20 days after germination, different parts of Seedlings (cotyledon, petioles and leaves) were transferred to regeneration media with different concentrations of BAP and TDZ (0 - 0.2 - 0.6 - and 1 mg/l). Two weeks later, the formation of buds was observed on different media and on different kinds of explants.

The first incidence of bud initiation was noted after 8 days in the form of small protuberances, then the meristematic domes of buds were observed after 12 days of culture, the formation of buds was recorded after 15 days and in many cases the developing buds fused together. The number and the size of shoot buds increased over the course of time (Fig.6, A, B, C and D).

The investigations showed that the bud formation occurred on medium MS with (TDZ and BAP) at different concentrations were effective to induce morphogenesis in *Artemisia annua*. The regeneration frequency depended on the kind of explants, source and the cytokinins concentrations. Similar results were obtained on plant propagation of *Artemisia vulgaris*( Sujatha and Kumari , 2007)

The morphogenetic capacity of different kinds of explants was increased with increasing cytokinins concentrations (Table 2). A maximum of shooting response was observed for medium containing 1mg/l of TDZ, with the highest number of shoots formed per explant among all treatments tested.

**Table. 2. Morphogenetic capacity in explants taken from seedlings and from greenhouse plants**

| Cytokinins used | mg/l | Regeneration percentage in explants taken from seedlings | | | Regeneration percentage in explants taken from greenhouse plants | | |
|---|---|---|---|---|---|---|---|
| | | Leaves | Petiole | Cotyledon | Leaves | Petiole | Internodes |
| BAP | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BAP | 0.2 | 16.6 a | 30 b | 33.3 b | 8 a | 30 b | 30 b |
| BAP | 0.6 | 50 c | 50 c | 33.3 b | 16.6 a | 30 b | 30 b |
| BAP | 1 | 66.6 d | 66.6 d | 50 c | 33.3 b | 33.3 b | 30 b |
| TDZ | 0.2 | 50 c | 66.6 d | 50 c | 8 a | 30 b | 30 b |
| TDZ | 0.6 | 83.3 e | 70 d | 60 d | 33.3 b | 30 b | 30 b |
| TDZ | 1 | **100** f | **100** f | **75** e | **50** c | **62** d | **42.6** c |

Treatments with the same letter are not significantly different at the 5% level (Duncan`s test).

The efficiency of shoots formation from different explants (petioles, leaves and internodes) taken off from mother plants was different under various cytokinins (BAP and TDZ) concentrations (Figs. 3 and 4). The capacity of bud formation of explants varied between different kinds of explants. (Table 2). A maximum shooting formation 61% from petiole and internodes was observed with the increasing of BAP and TDZ in media. The number of shoots formed increased also with the increasing of cytokinins concentrations. Media supplemented with BAP at the same concentrations gave a lesser morphogenetic response than media supplemented with TDZ at the same concentrations (Table 2).

**Fig. 3: Bud regeneration on petiole.**

**Fig.4: Bud regeneration on leaf**

Various plant genotypes required different optimum TDZ concentrations for morphogenesis (Chevreau *et al*., 1089; Shibli *et al*., 2000; ALMaarri and Toma, 2008). In our case the best results were obtained on medium with 1mg/l of TDZ. Similar results were obtained in *A.vulgaris* (Sujatha and Kumarry, 2007).

A very good relationship between the age and the source of explants and their morphogenetic capacity was observed. The explants from leaves and petioles of the plants seedlings induced higher regeneration percentage and number of buds compared to explants taken from the three months old mother plants.

Shoot obtained from different explants were transferred to multiplication medium (Table 1), where further auxiliary shoots were developed. Shoot subcultured every 4 weeks. The multiplication rate of shoots and the average number of shoots length increased with the number of subcultures (Fig.5).

**Fig. 5. bud multiplication**

The stimulating effect of subculture on shoot multiplication and elongation might be ascribed to their rejuvenation influence on *in vitro* culture. Several authors reported the multiplication of *A. annua* by using high concentration of BAP (Nam-cheol *et al*., 1992; Geng *et al*., 2001; Lee *et al*., 2003). Similar results were obtained on other species of Artemisia such *A. Scorpia* (Aslam *et al*., 2006), and *A ranunculus* (Mackay and Kitto, 1988). Liquid medium was used for mass propagation of *A. vulgaris*, which permit a big number of shoot proliferations during 4 weeks (Govindaraj *et al.,* 2008). However, morphological and physiological disorders such as hyperhydricity are commonly observed in plant and shoot produced in liquid culture, in particular in *Artemisia* sps. These abnormal changes might be related to stresses under micro environmental conditions. (Such as O2 and CO2 levels, shear and hydrodynamic forces) (Govindaraj *et al.*, 2008).

In our case, the vitrification in multiplication medium was frequently observed in *A. annua*. However it was reduced by transferring the shoots to medium containing half macro elements of MS.

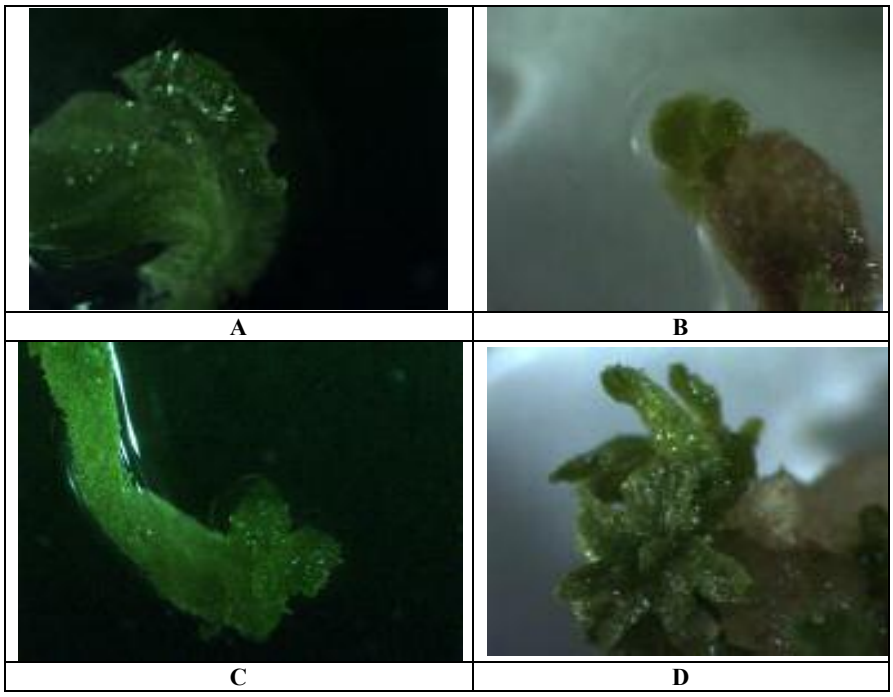


**Fig. 6. Bud initiation after 8 days (A), bud development after 12 (B), 15 days (C) and 20 days formation (D) (x 40 times).**

**Induction of adventitious roots *in vitro***:

The shoots formed on multiplication stage were desiccated and transferred to rooting medium (Table 1). Shoots were rooted after 20 days of culture with satisfactory rooting percentage (75%) and good root quality were observed on rooting medium containing 1mg/l of IBA. Rooted shoots are able to be transferred to greenhouse and grow normally .Similar results were obtained by using NAA and IAA for rooting of shoots in *A. annua* reported by Nam-Cheol *et al.,*1992).

It can be concluded that results obtained in this study, permit the development of a mass propagation protocol with a good multiplication rate and a high regeneration percentage from different kinds of explants, leaves, petioles, internodes and cotyledons of *A. annua.* Moreover the findings attained here might be useful in further research on genetic transformation and the regeneration plants true to type in *A.annua*.

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