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Effects of Plant Growth Regulators on Frequency Shoot Multiplication of an Important Ornamental and Medicinal Plant, Snowbush (*Breynia disticha*)

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ABSTRACT

The current research was conducted in Mansoura University, Faculty of Agriculture at Vegetable and Floriculture Department in the experimental station and tissue culture laboratory of. This study describes *in vitro* shoot multiplication from the nodal segments of the ornamental and medicinal shrub Snowbush (*Breynia disticha*). Multiple shoots were induced directly from the nodal explants on Murashige and Skoog (MS) medium fortified with different cytokinins, i.e., 6-benzyl aminopurine (BAP), kinetin (Kin), thidiazrone (TDZ), 2-isopentenyl adenine (2ip) and coconut water at different concentrations individually or in combination with auxins (indole-3-acetic acid, indole-3-butyric acid, α-naphthaleneacetic acid) or gibberellic acid (GA_3). BAP at a concentration of 0.5 mg/L was found to be the most effective cytokinin in inducing multiple shoots. Using auxins in combination with BAP was less effective on the frequency of shoot multiplication as compared to BAP alone. While, the frequency of shoot multiplication was markedly influenced by adding GA_3 to the media, since the highest percentage of shoot multiplication (100 %) as well as large number of shoots/ explant (4.16 shoots) were obtained with MS medium supplemented with BAP at 0.5 mg/L + GA_3 at 0.1 mg/L. Also, this medium succeeded to induce roots at the same stage with rooting percentage of 50 % and roots number of 4.15 roots. Mixture of soil: peatmoss (1: 1) was suitable planting substrate for hardening the rooted shoots and its use ensured high survival frequency (100 %).

INTRODUCTION

Snowbush (*Breynia disticha*) is an important ornamental and medicinal plant which belongs to family Phyllanthaceae. It is a rounded shrub has mottled, mall, multi-colored variegated leaves with green, red and white coloration leaves (Smith 1981; Lorence 1995). It is native of the western Pacific islands. Snowbush used as a specimen because of its beautiful foliage, forms a nice hedge garden, make for an accent in a shrub border and branches are flexible enough to drape over a wall (Edward 1999). Also, it had a high medicinal value as proved by Abid and Touqeer (2015). Ethnomedically, *Breynia disticha* is used in treating toothaches, tooth infections and headaches (Onyegbule *et al.* 2014). The extracts of *Breynia disticha* leaves contained sensible amounts of tannins, glycosides, alkaloids, flavonoids and starch with reasonable proteins and saponins. So, the extracts are natural analgesic, anti-oxidant, anti-inflammatory and antimicrobial agent also could have therapeutic potential in the handling of various chronic diseases such as malaria (Amadi *et al.* 2007; Onyegbule *et al.*, 2014; Jude *et al.*, 2015).

Recently, an increasing interest in cultivation and production of new ornamental and medicinal plants has been recognized in Egypt to cover the increasing demands of the local industries as well as, for export. In order to expand cultivation of *Breynia disticha*, the first step is the production of high quantities of genetically homogeneous plant material, since it is difficult to obtain seeds owing to Egyptian climate so usual multiplication procedure is vegetative (cutting). However, this plant has proved difficult to propagate (Vic 1987) because seeds take a long period for germination (about 5 month) and pretreatments are necessary for good germination also, stem cutting are tricky. So, these methods are not efficient enough to produce needed mass production.

The intensive multiplication by *in vitro* culture technique is a powerful tool for micro propagation and genetic improvement of the species. To the best of our knowledge, there are no reports on the micropropagation of *Breynia disticha*. Hence, the aim of the present work was to develop a methodology for *in vitro* multiplication of snowbush (*Breynia disticha*) and this paper describes the procedure that used to induce direct regeneration of shoots and subsequently plantlets production from nodal segments of this plant.

MATERIALS AND METHODS

1. Establishment stage.

Plant material and Surface sterilization.

Several newly healthy produced shoots of *Breynia disticha* were collected during the month of April (one month old) from adult donor plants growing in the farm of ornamental nursery, Mansoura University. Usually 10- 15 cm cutting were snipped early in the morning while the plants are fully turgid and forthwith placed in a jars filled with water until sterilization takes place. After removing the leaf blades with a scalpel leaving only a small basal section, the shoots were cut into nodal segments about 1–1.5 cm long and consisting of a single bud. The clean-up process of excised nodal segments involves washed with cold running tap water along with surfactant agent such as Tween 20 for 60 minutes. Thereafter, an experiment was carried out to investigate the effect of sterilization with sodium hypochlorite at different concentrations (i.e., 2 and 3 %) for three different periods (i.e., 10, 15,18 and 20 minutes). During this step of surface sterilization, container which contained the explants was shaken as much as possible. Then, the explants were thoroughly rinsed 3 times in sterile distilled water for 3 minutes each.

After surface sterilization, the explants were cultured into 250 ml sterilized glass jars, contained 30 ml of Murashig and Skoog (1962) free nutrient medium (one explant in each jar) and directly covered with polyproplene closures. Each treatment consisted of 4 replicates included 12 jars using a factorial experiment in a randomized complete block design.

Culture media and conditions.

In all experiments the most commonly basic tissue culture media and is suitable for many applications Murashige and Skoog (1962) nutrient medium was used. The medium was fortified with sucrose at 30 g/L and 7g agar /L (w/v) was used to solidify tissue culture media into a gel. Before agar was added to the media and after adding growth regulators adjusting the pH of the medium between

5.7 and 5.8 was done by using HCl to raise pH or NaOH to lower it. All tested media were sterilizing by autoclaving at 121 °C with a pressure of 15 psi for 15 minutes.

After culturing the explants on the glassware contained 25 ml of medium under the sterile transfer hood, they were moved to plant growth room with temperature of 25 ± 2°C under cool white fluorescent lights of 2500 Lux for photoperiod of 16/8 h (light/dark).

2. Multiplication stage:

A single nodes along with one axillary bud were cultured on MS medium fortified with different cytokinin types, i.e., BAP (6-benzyl aminopurine), Kin (kinetin), TDZ (Thidiazuron), 2ip (2-isopentenyl adenine) and coconut milk. The first four cytokinin types were added to the media at three different concentrations (0.5, 1.0 and 2.0 mg/L) for each substance while the coconut milk was added at concentrations of 2, 4 and 6 %.

The best Cytokinin type and concentration was then tested in combination with different auxin types, i.e., indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) at different concentrations (0.05, 0.1, or 0.25 mg /L) and gibberellic acid (GA3) at concentrations of 0.05, 0.1, 0.25 or 0.5 mg /L for determination of the best *in vitro* multiplication conditions. For all experiments each treatment consisted of 4 replicates included 12 jars using a completely randomized design.

3. Acclimatization stage:

The *in vitro* rooted plantlets were taken out from the cultured glassware; the roots were cleaned up with warm water many times to remove all vestiges of culture media. Then solo rooted plantlets were placed directly into moistened soilless growing medium [soil: peatmoss (1:1 v/v)] contained in plastic pots which placed in small glass container cover with clear plastic sheets to maintain humidity. After four weeks the sheet was gradually removed to lower humidity and plantlets gradually were exposed to higher light intensities and temperature to promote vigorous growth.

Statistical analysis:

Data of all experiment was subjected to analysis of variance (ANOVA) by the general linear models (GLMs) procedure using (SAS) Statistical Analysis System (2000). Mean comparisons were performed using the least significant difference (LSD) method according to (Gomez and Gomez, 1984). A significance level of 5 % was used for all statistical analyses.

RESULTS AND DISCUSSION

1- Effect of sterilization treatments on nodal segments of *Breynia disticha*.

In the present study a comparison was done among two concentrations of sodium hypochlorite (2 and 3 %) with three time durations (10, 15 and 18 minutes) on nodal segments of *Breynia disticha*. To study the effect of sterilization treatments, four parameters were evaluated, i.e., survival percentage, contamination percentage, shooting percentage and shoots number per explant as shown in Table (1).

Concerning the effect of sterilizations treatment on nodal segments of *Breynia disticha*, data in Table (1) showed that sodium hypochlorite at concentration of 3 % with the three different durations have the upper hand in survival percentage when compared with sodium hypochlorite at concentration of 2 %. The highest significant survival percentage (100 %) was achieved with sodium hypochlorite at 3 % for 18 minutes (Fig. 1). While, the concentration of 2 % sodium hypochlorite at the same duration gave only 41.7 % survival percentage and 58.3 % contamination percentage. Using sodium hypochlorite at 2 % significantly increased the rate of contamination to 100 % at the duration of 10 minutes. Also, it is worth to mention that exceeding sterilizing duration up to 20 minutes was harmful for the treated tissue and increased mortality percentage of nodal segments to 100 % with concentration of 3 % sodium hypochlorite (data not shown in table).

Figure 1. *Breynia disticha* shoot in MS medium without plant growth regulators after four weeks of surface sterilization with sodium hypochlorite at 3 % for 15 minutes.

So, preparing sterile explants is difficult because the tissue must be treated with disinfectants that are effective in destroying any microbial contamination without harming the explant tissue. It is important to be cautious that a surface sterilization is also toxic to the explants tissue, therefore concentration of the sterilizing agent and duration of the treatment should be optimum to minimize tissue mortality of the explants due to over sterilization. Also, it was observed from the data in the same table that culturing nodal segments on MS medium free hormone failed to induce multiple shoots it just gave one shoot per explant. The obtained results are in harmony with a number of reports (Cecilia *et al.* 2006; Brian *et al.* 2008; Frabetti *et al.* 2009) for sterilization of different shrub explants with NaOCl at different concentrations.

Table 1. Effect of sterilization treatments on nodal segments of *Breynia disticha*.

Treatments		Growth measurements			
Time (min.)	NaOCl conc. %	Survival %	Contamination %	Shooting %	Shoots number/ explant
10	2	0.0	100.0	0.0	0.00
	3	58.3	41.7	16.7	1.00
15	2	33.3	66.7	16.7	1.00
	3	75.0	25.0	16.7	1.00
18	2	41.7	58.3	16.7	1.00
	3	100	0.0	33.3	1.00
L.S.D. at 5 %		18.9	17.4	14.9	*

2- Effect of cytokinin types on nodal segments of *Breynia disticha*.

Many different factors of the *in vitro* environmental and the explants affect success *in vitro*, but the type,

concentration and duration of exposure to plant growth regulators typically have the most profound effect. The choice of plant growth regulator to use is predicated on what outcome is desired. In this experiment the function of cytokinin types at different concetrations activity on shoot proliferation in cultures of nodal explants was shown in Table (2).

Of the five cytokinins tested, BAP at the three different concentrations was most effective in inducing bud break. One should be aware of the fact that, although a given cytokinin may not work well with certain species; it may be quite effective in others. In the herein study, it was BAP at the concentration of 2 mg/L the treatment which gave the highest responded explants percentage (100 %) but with shoot number of 1.33 per explant. While, the explants cultured on medium fortified with BAP at the concentration of 0.5 mg/L gave the highest significant shoot number per explant (3.67 shoots/ explant) with responded explants percentage of 91.7 % and average shoot length of 2.66 cm which possessed 5.38 leaves per shoot (Fig. 2A). The next positive effect for shoots number per explant was obtained with BAP at the concentration of 1 mg/L (2.29 shoots/ explant) with responded explants percentage of 91.7 % and average shoot length of 1.81 cm which possessed 4.15 leaves per shoot. The more efficiency of BAP in induction of multiple shoot formation than other cytokinins has been identified in several medicinal plants (Kozomara *et al.* 2008; Nassem and Mohammed 2010). For shoot length and leaves number per shoot, the medium fortified with Kin at concentration of 2 mg/L tabulated the longest shoot length of 3.31 cm and the highest leaves number per shoot of 5.88 leaves (Fig. 2B), followed by 3.10 cm and leaves number of 5.63 leaves per shoot when using Coconut milk at 2 % and there were no significant differences between them.

On the other hand, TDZ at 0.5 - 2.0 mg/L was found to be the least effective of all tested cytokinins. Although it successes to rich responded explants percentage of 75 % on MS containing TDZ at 0.5 mg/L, but it failed to induce multiple shoots it just gave one shoot per explant and these shoots failed to elongate and were often fascinated specially at the higher concentration (2.0 mg/L) which significantly tabulated the lowest shoot length of 3.31 cm with leaves number of 2.73 leaves per shoot as shown in Fig. 2C. The formation of stunted shoots or fasciations of shoots on TDZ supplemented medium has been reported in several studies (Shoo and Chand 1998; Gabr 2004). The inhibition of shoot elongation may be due to the high cytokinin activity of TDZ where as the presence of a phenyl group in TDZ may be the possible cause of shoot bud fasciation (Huetteman and Preece 1993).

Generally, in the whole plant cytokinins play a role in a variety of processes including development of chloroplasts, delay of senescence, cell division and differentiation, vascular development, resource uptake and allocation, nodulation in leguminous species as well as initiation and development of shoots. One of the most commonly used cytokinin is BA. This cytokinin has been proven to be effective in stimulating proliferation of shoot and formation of callus in a wide range of herbaceous and woody species (Trigiano and Gray 2011).

Table 2. Effect of cytokinin types at different concentrations on nodal segments of *Breynia disticha* after 4 weeks of culture.

Treatments		Responded explants %	Shoots Number/ Explant	Shoot Length (cm)	Leaves Number/ shoot
cytokinin type	cytokinin conc.				
BAP	0.5 mg/L	91.7	3.67	2.66	5.38
	1.0 mg/L	91.7	2.29	1.81	4.15
	2.0 mg/L	100	1.33	0.90	2.50
Kin	0.5 mg/L	50.0	1.63	2.15	4.23
	1.0 mg/L	75.0	1.21	2.71	5.35
	2.0 mg/L	83.3	1.83	3.31	5.88
TDZ	0.5 mg/L	75.0	1.00	1.05	2.60
	1.0 mg/L	50.0	1.00	1.00	2.75
	2.0 mg/L	50.0	1.00	0.83	2.73
2-ip	0.5 mg/L	83.3	2.08	2.80	5.22
	1.0 mg/L	83.3	1.58	1.49	3.67
	2.0 mg/L	66.7	1.08	1.98	4.65
Coconut Milk	2 %	83.3	1.99	3.10	5.63
	4 %	83.3	1.54	1.81	3.83
	6 %	50.0	1.38	1.21	2.62
L.S.D. at 0.05		29.3	0.46	0.41	0.81

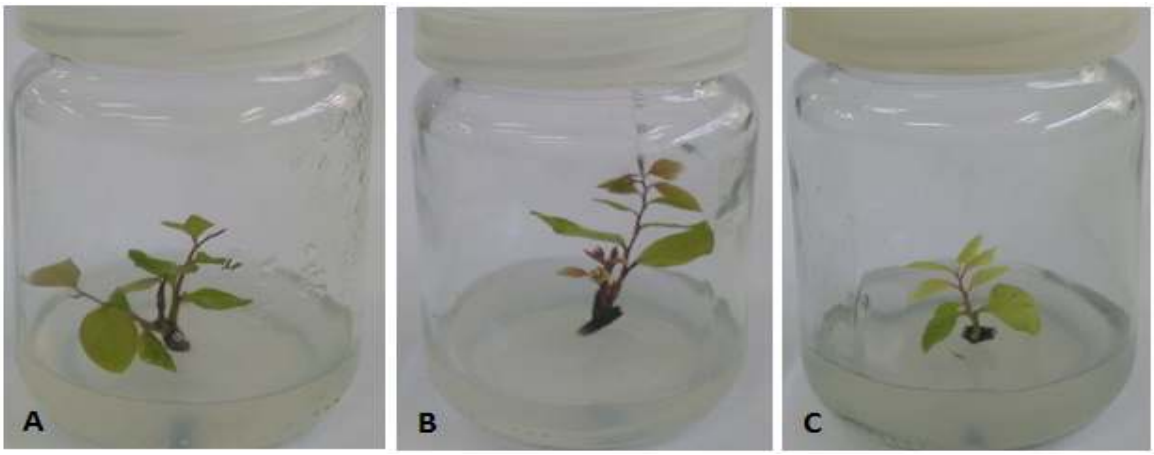

Figure 2. *In vitro* culture of *Breynia disticha* nodal segments on MS medium. A) Multiple shoots, obtained with 0.5 mg/L of BAP. B) Multiple shoots, obtained with Kin at 2 mg/L. C) Multiple shoots, obtained with 2 mg/L of TDZ.

3- Effect of different auxin types at different concentrations in combination with BA on development of *Breynia disticha* nodal segments.

The effect of combination between auxins and cytokinins on nodal segments of *Breynia disticha* to improve the multiplication was done by using three different auxins (i.e., NAA, IBA and IAA) at different concentrations (0.05, 0.1 and 0.25 mg/L) with BA at 0.5 mg/ L. The recorded results in Table (3) cleared that IBA had the upper hand in the respect of responded explants percentage when compared with the other two auxins at the same concentration. MS medium supplemented with BAP at 0.5 mg/L and IBA at 0.25 mg/L recorded the highest responded explants percentage 91.7 %. While, media supplemented with BAP at 0.5 mg/L in combination with IAA at the three different concentrations recorded the lowest responded explants percentage, since the lowest percentage (41.7 %) was obtained with the nutrient medium fortified with IAA at 0.05 mg/ L and BAP at 0.5 mg/L.

The best interaction effect for the induction multiple shoots with average of 2.85 shoots per explant was obtained with medium containing BAP at 0.5 mg/ L and IBA at 0.25 mg/L with a significant differences compared to all other treatments, while the lowest multiple shoots (1.42 shoots/ explant) was recorded with BAP at 0.5 mg/ L and NAA at 0.25 mg/L (Fig. 3A&B). For shoot length, the greatest shoot length value (2.69 cm) was obtained with BAP containing medium supplied with IBA at 0.1 mg/L. On contrast, using medium fortified with BAP at 0.5mg/L and NAA at 0.1 mg/L recorded the least value of 1.97 cm for the shoot length.

It was a matter of importance to mention that MS media supplemented with combination of BAP and NAA at different concentrations had a marked effect on root initiation of the multiple shoots in this experiment, while IBA and NAA completely failed to induce rooting of multiple shoots. MS medium with BAP at 0.5mg/L and NAA at 0.1 mg/L was the most efficient treatments, since the highest rooting percentage of 66.7 % and the highest significant roots number of 8.25

roots/ explant were recorded but the roots was always very short and thick without secondary roots and roots hairs (Fig. 3C).

Generally, cytokinins are the plant growth regulators mainly associated with shoot proliferation. But, usually a combination of two or more different types of growth regulators is required for successful *in vitro* shoot proliferation, interaction between cytokinin and auxin considered to be the most important for regulating plant growth (Evans *et al.* 1981). In the present investigation it was observed that BAP was the most effective cytokinin in induction multiple shoots and the response of explants decreased after adding different auxins (NAA, IBA and IAA) to the media. Similar observation was made in *Jatropha curcas* (Misra *et al.* 2010).

Table 3. Effect of different auxin types at different concentrations in combination with BA at 0.5 mg/L on development of *Breynia disticha* nodal segments.

Treatments		Responded explants %	Shoots Number/ explant	Shoot Length (cm)	Rooting %	Roots number/ explant
Auxin type	Auxin conc. mg/L					
	0.05	75.0	1.62	2.61	41.7	3.92
NAA	0.1	75.0	1.54	2.48	66.7	8.25
	0.25	83.3	1.42	2.10	50.0	6.00
	0.05	83.3	2.17	2.36	0.0	0.0
IBA	0.1	83.3	2.42	2.69	0.0	0.0
	0.25	91.7	2.85	1.97	0.0	0.0
	0.05	41.7	1.75	2.13	0.0	0.0
IAA	0.1	50.0	2.00	2.13	0.0	0.0
	0.25	66.7	2.13	2.53	0.0	0.0
L.S.D. at 0.05		27.6	0.35	0.52	23.6	0.69

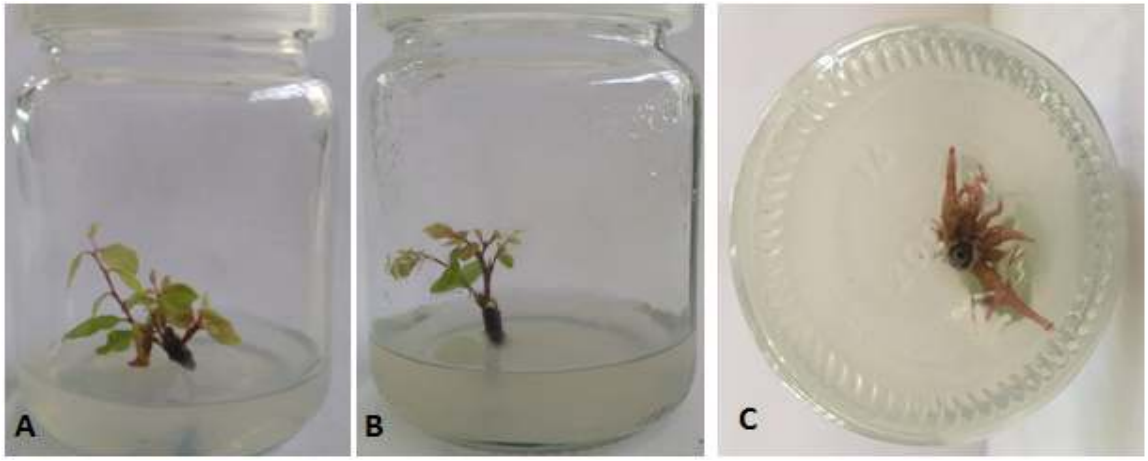

Figure 3. *In vitro* culture of *Breynia disticha* nodal segments on MS medium. A) Multiple shoots, obtained with BAP at 0.5 mg/ L and IBA at 0.25 mg/L. B) Multiple shoots, obtained with BAP at 0.5 mg/ L and NAA at 0.25 mg/L. C) Roots, obtained with BAP at 0.5 mg/ L and NAA at 0.1 mg/L.

4- Effect of gibberellic acid at different concentrations in combination with BA on development of *Breynia disticha* nodal segments.

In some cases gibberellic acid may be used, in place of auxin, plus a cytokinin for shoot induction. The addition of GA_3 to media may also improve the survival of meristem explants, but this response is quite variable (Yadav and Tyagi, 2006). The effect of interaction between BAP at 0.5 mg/L and GA_3 at four concentrations (0.05, 0.1, 0.25 and 0.5 mg/L) on development of *Breynia disticha* nodal segments was shown in Table (4).

Concerning the responded explants percentage, data showed that application of GA_3 at concentration of 0.1 mg/ L to the MS medium supplemented with BAP had a positive effect on this character since it recorded 100 % responded explants. The next effect in that respect was on media had GA_3 at 0.05 or 0.25 mg/L and BAP at 0.5 g/L with the value of 83.3 % for both of them but no significant differences among all treatments were detected. As for shoots number per explant, results clearly showed that the highest significant shoots number per explant (4.16 shoots) was obtained with the same treatment (BAP at 0.5 mg/L + GA_3 at 0.1 mg/L) which recorded the highest responded explants percentage as shown in Fig. 4A. Also, the same treatment succeed to record the highest shoot length of 2.44 cm but no significant differences among all treatments were detected except the lowest concentration of GA_3 (0.05 mg /L) with BAP which recorded the lowest significant shoot length (1.38 cm).

It was a matter of importance to mention that MS media supplemented with combination of BAP and GA_3 at different concentrations had a marked effect on root initiation of the multiple shoots in this experiment. MS medium with BAP at 0.5 mg/L and GA_3 at 0.1 mg/L was the most efficient treatments, since the highest rooting percentage of 50 % and the highest significant roots numbers of 4.15 roots were recorded. On the other hand, the weakest effect for roots number was with explants cultured on medium fortified with the two combined substance BAP at 0.5 mg/L and GA_3 at 0.05 g/L, since it was 1.5 roots number per explant. The roots which obtained in this trial were always medium to long in length and thin with secondary roots and roots hairs (Fig. 4B).

In the present experiment the promotive effect of the combination between BAP and GA_3 on shoot multiplication of *Breynia disticha* was in agreement with Sahoo and Chand (1998) on *Vitex negundo* and Gisele and Thomas (2005) on *Viburnum odoratissimum*. They indicated that BA – GA_3 coupling had synergistic effect on multiple shoot formation and increased number of shoots per explants. The positive effect may be due to that Gibberellins (GAs) can promote a wide range of physiological activities in plants, including fruit development, seed germination, dormancy breakage, flowering and stem elongation through the increase of cell division, cell wall formation and expansion (Huttly and Phillips 1995).

Concerning the three previous cited experiments, it could be concluded that the best treatment in induction multiple shoots for *Breynia disticha* was MS medium fortified with BA at 0.5 mg/L and GA_3 at 0.1 mg/L, since it succeeded in realizing the highest values of shoots number per explant (4.16 shoots) with responded explants percentage of 100 % and average shoot length of 2.44 cm which also possessed rooting percentage of 50 % and roots number of 4.15 roots.

The *in vitro* rooted plantlets were taken out from the cultured glassware; the roots were cleaned up with warm water many times to remove all vestiges of culture media. Then solo rooted plantlets were placed directly into moistened soilless growing medium [soil: peatmoss (1:1 v/v)] contained in plastic pots. About 100 % plantlets were surviving one month after transfer.

Table 4. Effect of gibberelic acid at different concentrations in combination with BA at 0.5 mg/L on development of *Breynia disticha* nodal segments.

Treatments		Responded explants %	Shoots Number/ Explant	Shoot Length (cm)	Rooting %	Roots number/ explant
	Conc. mg/L					
GA_3	0.05	83.3	2.41	1.38	41.7	1.50
	0.1	100	4.16	3.26	50.0	4.15
	0.25	83.3	1.16	2.38	33.3	1.62
	0.5	75.0	1.08	2.413	33.3	2.25
L.S.D. at 0.05		28.4	0.72	0.57	22.2	0.85

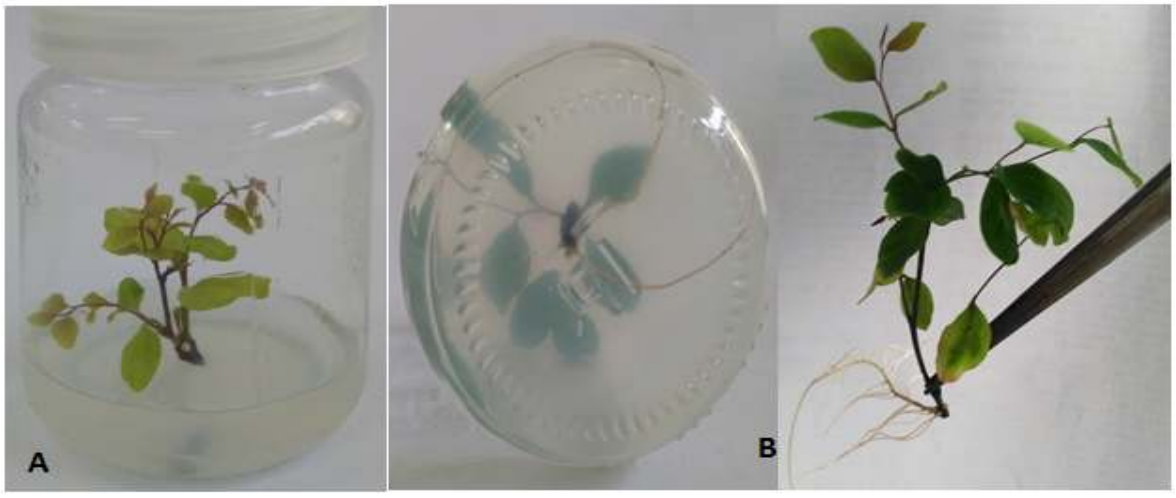

Figure 4. *In vitro* culture of *Breynia disticha* nodal segments on MS medium. A) Multiple shoots, obtained with BAP at 0.5 mg/ L and GA_3 at 0.1 mg/L. B) Rooted shoots, obtained with BAP at 0.5 mg/ L and GA_3 at 0.1 mg/L.

Figure 5. Plantlets of *Breynia disticha* produced from tissue culture after six weeks from acclimatization.

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تأثير منظمات النمو النباتية على معدل تضاعف الأفرع لنبات زينة وطبي هام (*Breynia disticha*)

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اجري هذا البحث بجامعة المنصورة ـ كلية الزراعة في قسم الخضر و الزينة بمحطة تجارب معمل زراعة الانسجة. تصف الدراسة تضاعف الأفرع معمليا باستخدام قطع ساقية برعمية لنبات زينة و طبي شجيري (*Breynia disticha*).الأفرع المتضاعفة تم حثها مباشرة من قطع ساقية برعمية علي بيئة موراشيج و سكوج محتوية علي سيتوكينينات مختلفة و هي البنزيل أمينو بيورين ـ الكينتين ـ الثيديازيورون- ايزوبنتيل أدينين و ماء جوز الهند بتركيزات مختلفة اما منفرده او في توليفات مع الاوكسينات (اندول حمض البيوتريك ـ نفثالين حامض الخليك ـ اندول حامض الخليك) و حمض الجبريليك. وجد ان البنزيل أمينو بيورين بتركيز ٠,٥ مللجم/ لتر كان اكثر السيتوكينينات فعالية في حدوث تضاعف للأفرع. وجد ان استخدام الأوكسينات في توليفات مع البنزيل أمينو بيورين كان أقل فعالية في التأثير على معدل تضاعف الأفرع مقارنة باستخدام البنزيل أمينو بيورين منفردا. بينما تأثر معدل تضاعف الأفرع بشكل ملحوظ عند اضافة حمض الجبريليك مع البنزيل أمينو بيورين حيث كانت أعلى نسبة تضاعف للأفرع ١٠٠ % و أكبر عدد أفرع ٤.١٦ فرع تم الحصول عليها من بيئة موراشيج و سكوج مزودة بي البنزيل امينوبيورين ٠,٥ مللجم/ لتر و حمض الجبريليك ٠.١ مللجم/ لتر. أيضا هذه البيئة نجحت في تكوين جذور في نفس المرحلة بنسبة تجذير ٥٠ % و عدد جذور ٤.١٥ جذر. خليط من التربة و البيتموس بنسبة (١:١) كانت مناسبة لأقلمة الأفرع ذات الجذور.