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ARTICLE

Production of indole acetic acid (bioauxin) from *Azotobacter* sp. isolate and its effect on callus induction of *Dieffenbachia maculata* cv. Marianne

Mohammed Elsayed El-Mahrouk[1]*, E B A Belal[2]

[1]Department of Horticulture, Faculty of Agriculture, Kafr El-sheikh University, Kafr El-sheikh, Egypt, [2]Department of Agriculture Botany, Faculty of Agriculture, Kafr El-sheikh University, Kafr El-sheikh,Egypt

ABSTRACT Indole-3-acetic acid (IAA) in the supernatant of a culture from the strains, *Rhizobium leguminosarum biovar viciae*, *Bradyrhizobium japonicum*, *Pseudomonas* sp. and *Azotobacter* sp was detected. *Azotobacter* sp yielded the highest concentrations of IAA. It was shown that the indole-3-acetic acid (IAA) was induced by the presence of tryptophan, which is used as inducer because the plant provide the bacteria with tryptophan under natural conditions. The highest concentration of IAA was produced by *Azotobacter* sp.(A1) at the end of the logarithmic phase (after 3days). The results obtained in this work provide useful information about the production behavior of IAA under the optimal conditions(temperature 30°C and pH 7) which is of importance for the application in production *Dieffenbachia maculat* cv. Marianne plants by using tissue culture technique. This work was also conducted to study the effect of some growth regulators such as 10 mg/l IAA (synthetic), 5 mg/l BA and 10 mg/l IAA (bioauxin) on callus formation of *Dieffenbachia maculat* cv. Marianne shoot tips and internodal segments were taken from sterilized shoot and cultured on MS medium supplemented with 6 different treatments from growth regulators. Explants cultured on MS medium supplemented with either 10 mg/l IAA +5 mg/l BA or 10 mg/l bioauxin + 5 mg/l BA had the highest callus percentage 97.22 and 93.94%, respectively. MS medium supplemented with 2 mg/l BA + 0.06 mg/l BA was used for callus differentiation.
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KEY WORDS

Dieffenbachia
callus formation
indole acetic acid
bezial adenine
Azotobacter sp.

The ability to produce the plant hormone indole-3-acetic acid (IAA) is widespread among soil, epiphytic, and tissue-colonizing bacteria (Costacurta and Vanderleyden 1995; Pat-ten and Glick 1996; Barazani and Friedman 1999). These genera of bacteria comprises *Azospirillum, Azotobacter* sp *Rhizobium, Bradyrhizobium, Enterobacter, Xanthomonas, Klebsiella* sp., *Serratia* sp., *Pseudomonas* spp., cyanobacteria and sulfur oxidizing bacteria. These bacteria have shown to enhance plant growth, by promoting the out-break of secondary roots, acting as protectors against phytopathogenic microorganisms via plant hormones release and siderophores (Tien et al. 1979; Fett et al. 1987; Zimmer and Bothe 1988; Sekine et al. 1989; Minamisawa and Fukai 1991; Gamliel and Katan 1992; Amstroen et al. 1993; Bar and Okon 1993; Glick 1995; Patten and Glick 1996). Other benefits may include suppression of plant defense, which facilitates bacterial invasion (Robinette and Matthysse 1990).

Dieffenbachia maculata cv. Marianne belongs to the family Araceae, it is attractive foliage plant and is generally propagated vegetatively by cuttings. However, the traditional propagation using cutting is sometimes encountered with various difficulties such as fungal, bacterial and viral diseases (Chose 1987). Aroids show a high incidence of aberrant plants when propagated by tissue culture (Debergh and Maene 1981). Cytokinins or auxins are necessary in callus formation and differentiation. Cytokinins are used mostly for induce shoot bud induction, stimulate cell division, both formation and growth axillary and adventitious shoot in tissue culture. On the other hand, auxins are known to affect many process

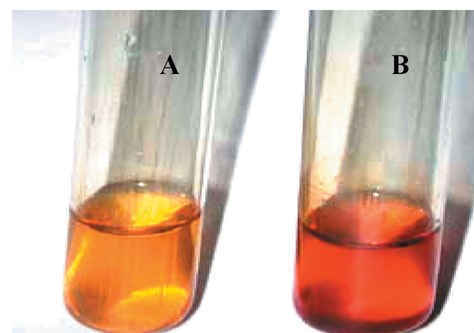

Figure 1. Production of indole-3-acetic acid by *Azotobacter* sp.(A1) , where A without tryptophan, B with tryptophan.

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*Corresponding author. E-mail: threemelmahrouk@yahoo.com

Table 1. Indole -3-acetic acid concentration found in liquid medium from the cultures of the most productive strains.

Source of microorganisms	Isolates	Indole-3-acetic acid (mg /ml)	
		Liquid medium without tryptophan	Liquid medium with tryptophan
Soil samples	*Azotobacter* sp. (A1)	-	54
	Azotobacter sp. (A2)	-	52
	Azotobacter sp. (A3)	-	48.1
	Azotobacter sp. (A4)	-	44.1
	Azotobacter sp. (A5)	-	30
	Azotobacter sp. (A6)	-	20
	Azotobacter sp. (A7)	-	16.4
	Azotobacter sp. (A8)	-	16.4
	Azotobacter sp. (A9)	-	14
	Azotobacter sp. (A10)	-	12
	Pseudomonas sp. (P1)	-	15
	Pseudomonas sp. (P2)	-	12
	Pseudomonas sp. (P3)	-	15
Root nodules	*Pseudomonas* sp. (P4)	-	7
	R. leg. biovar viciae (R1)	-	30
	R. leg. biovar viciae (R2)	-	15
	R. leg. biovar viciae (R3)	-	10
	B. japonicum (B1)	-	12
	B. japonicum (B2)	-	30
	B. japonicum (B3)	-	15

in plant including cell elongation and adventitious root formaton (Trigiano and Gray 1996). The presence of either N^6(A-isopentenyl) adenine (2ip) or kinetin in the medium was a prerequisite by Dieffenbachia for shoot formation *in Vitro* (Voyiatazi and Voyiatzis 1989) axillary meristem activation of mammillaria San-angelensis was observed only in the presence of IAA (Rubluo et al. 2002).

The aim of the present study was designed to produce of biouxin (IAA) from *Azotobacter sp.* (A1) and asses its influence on the callus induction of *Dieffenbachia* cv. Marianne

Materials and Methods

Sampling

Microorganisms were collected from rhizosphere of twenty maize plants as well as from active nodules initiated on healthy pea and soybean plants.

Microorganisms isolation and identification

Samples of 10 g of rhizosphere (roots and soil) were shaken with 90 ml of culture king,s B medium to isolate *Pseudomonas* sp. Ahmad et al. (2005), Jensen's liquid medium was used for *Azotobacter* sp. (Jensen 1951). The medium was shaked at 30°C and 150 rpm for 30 min, after that dilution series were prepared in glass tube containing 9 ml from each of the used media up to 1: 10^7. One hundred µl from the three later dilution from series were spreaded on plates containing the same medium + tryptophan (0.1g/l) by using drigalisky triangle. The plates were sealed in polyethylene bags and were incubated at 30°C for 4 days monitored for appearance of colonies. Single colonies growing on these dilution plates were picked up and maintained on the same medium to use for further studies. The rhizobial isolates (*Rhizobium leguminosarum* or *Bradyrhizobium japonicum*) were isolated from nodules on Yeast extract mannitol agar (YMA) supplemented with 0.3% calcium carbonate tryptophan (0.1g/l) using the methods described by Vincent (1970), Belal et al. (1996) and El-Nady and Belal (2005). Identification of grown isolated colonies was based on morphological, biochemical and culturing characteristics according to Bergy's manual of systematic bacteriology (1984) and Somasegaran and Hoben (1985).

Indole-3-acetic acid production

IAA was obtained from *R. leguminosarum* and *B. japonicum* in YM lquid medium with 0.1 g/l tryptophan tryptophan and from *Azotobacter* sp. in Jensen's liquid medium supplemented with 0.1g/l tryptophan as well as from *Pseudomonas* sp. in King's B medium with 0.1 g/l tryptophan. All strains were incubated at 30°C and 150 rpm for 3-5 days

Colorimetric analysis

After centrifugation (6000 rpm for 30 min), supernatant was used to determine indole-3-acetic acid (IAA) by the method described by Glickman and Dessaux (1995) and Ahmad et al. (2005). The developed (30 min) color was measured by spectrophotometer at 530 nm. Concentrations were calculated from an adjusted calibration curve.

Cultivation of *Azotobacter* sp. in Jensen's liquid medium for indole-3-acetic acid production

One hundred ml of Jensen's liquid medium supplemented with tryptophan(0.1 g/l) and 1ml of a cell suspension of *Azotobacter* sp. (Jensen's broth medium, 10^7 cfu/ml, incubated at 30°C and 150 rpm for 3 days). The culture was incubated at 30°C and 150 rpm for 3 days. The production of indole-3-acetic acid was determined daily by the described method (Ahmad et al. 2005). The growth representing in intracellular protein content for bacterial isolate was determined in each treatment as intracellular protein content (µg/ml). The bacteria cells were digested as described by Belal (2003) and the protein content was determined according to the method described by Lowry et al. (1951) using bovine serum albumin as standard protein.

Effect of pH and temperature on production of indole-3-acetic acid by *Azotobacter* sp.(A1)

One hundred ml of Jensen's liquid medium supplemented with 0.1 g/l tryptophan was used to determine the effect of

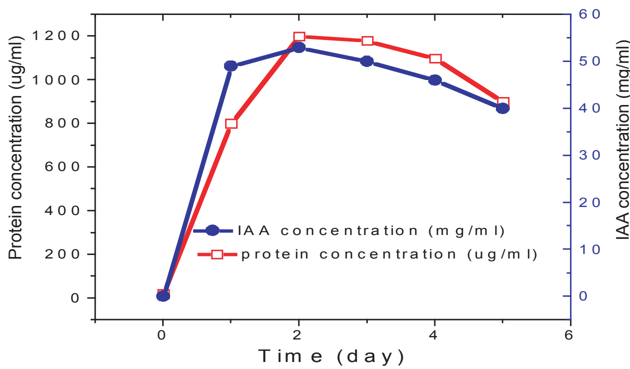

Figure 2. IAA production by *Azotobacter* sp. (A1) isolate in submerged culture with tryptophan.

temperature and pH on production of indole-3-acetic acid by *Azotobacter* sp.(A1).The medium was inoculated by 1ml (10^8 cfu/ml) of culture of *Azotobacter* sp.(A1) strain. The experiments were carried out at pH 6, 7 and 8 and the culture was incubated at 30°C and 150 rpm for 3 days. To determine the optimum temperature Ashby broth medium at pH 7 was incubated at 20, 30 and 40°C and 150 rpm for 3 days.

Callus induction

Shoot tips and internodal segments of Marianne cv. plants were used for callus induction. Surface sterilization was carried out by washing explants thoroughly with mild liquid detergent under a running tap water for 30 min. Then, under cabinet laminar flow, the explants were surface sterilized by dipping in 70% alcohol (ethanol) for two min. and later in 0.1% mercuric chloride with a few drops of wetting agent Tween-20 for 20 min. After sterilization, explants were rinsed four times with a sterilized water, 5 min for each time (Dodds and Roberts 1985; Kyte 1987 and Torres 1989). The sterilized explants were gently held with a fine forceps. Shoot tips and internodal segments of Marianne cv. plants were cultured on MS medium supplemented with 6 different treatments from growth regulators (BA, IAA and bioauxin) as follows:

Control (free growth regulator)
10 mg/l (synthetic) IAA
5 mg/l BA
10 mg/l (bioauxin) IAA
10 mg/l (synthetic) IAA + 5 mg/l BA
10 mg/l (bioauxin) IAA + 5 mg/l BA.

The medium pH was adjusted to 5.7 with 1N NaOH and 1N HC after activated charcoal at 2 g/l was added. Agar (8 g/l) was added before autoclaving. The medium was autoclaved for 15 min. at 121°C and 1.1 kg/cm^2, after that 15 ml medium was dispensed into sterilized Petri-dishes (7 cm). The experiment included 6 different treatments. Each treatment consisted of 8 Petri-dishes in a randomized complete design. The cultures were incubated under dark condition at 26 ± 2°C for two months, where the explants were subcultured after one month. The following data were recorded after two months of culture:

Callus frequency percentage
Callus fresh weight (g)
Callus diameter (cm).

Data were tested by analysis of variance and Dancan's multiple range test was used for the comparison among the treatment means (Duncan 1955).

Callus differentiation

The derived callus from the 6 different treatments of induction medium were cultured on the differentiation medium supposed by (Chamail et al. 1999) which consisted of MS basal nutrient medium supplemented with BA at 2 mg/l and IBA at 0.06 mg/l for one month. The cultures were incubated under light intensity 2000 Lux for 16 hrs photoperiod at 25 ± 2°C. After one month of culture on differentiation medium, callus were transferred to MS hormones free medium for two months. Number of plantlets/callus was recorded.

Analysis of data

Data were tested by analysis of variance and Duncan's multiple range test was used for the comparison among the treatment means (Duncan 1955).

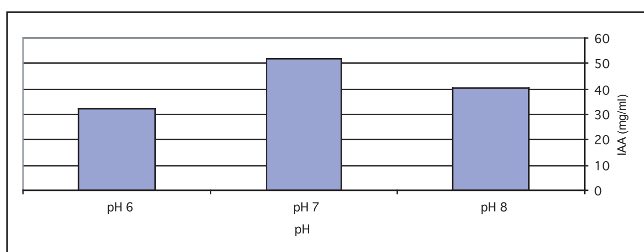

Figure 3. Effect of pH on production IAA by *Azotobacter* sp. (A1).

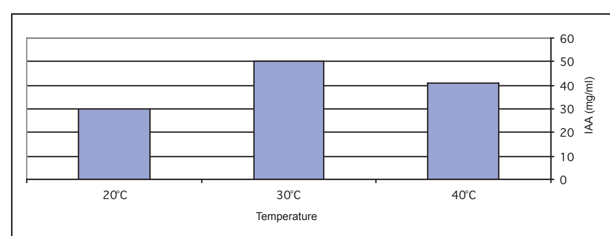

Figure 4. Effect of Temperature on production IAA by *Azotobacter* sp. (A1).

Results and Discussion

Isolation of indole -3-acetic acid producing by bacterial isolates

Two different sources used to isolate the IAA - producing isolates were evaluated in the present work. The first source was soil maize rhizosphere samples which were collected from different locations in Kafr El-Sheikh Governorate, Egypt. The second source was active nodules initiated on healthy pea and soybean plants. By using specific medium supplemented with tryptophan (0.1 g/l) for each isolates, a total of 20 morphologically different microorganisms were isolated from the both described microbial sources. Fourteen of 20 bacterial isolates were isolated from soil samples. Ten of 20 bacterial isolates belong to the genus *Azotobacter* and 4 of 20 belong to genus *Pseudomonas.* Six of 20 were isolated from nodules where 3 isolates belong to *Rhizobium leguminosarum biovar viciae* (isolated from pea plants) and the other third isolates belong to *Bradyrhizobium japonicum* (isolated from soybean plants; Tables 1). All twenty treated strains (10 *Azotobacter* sp., 4 *Pseudomon*as sp., 3 *R. leguminosarum biovar viciae* and 3 *B. japonicum*) in a culture medium containing tryptophan as inducer for IAA, produced IAA, as detected by Glickmann and Dessaux (1995), while the same strains did not produce IAA in the same medium without tryptophan. Most species use tryptophan to produce indole-3-acetic acid (IAA), mainly through the indole-3-pyruvic acid and tryptamine pathways (Bar and Okon 1993). Figure 1 shows samples of the attained solutions of this compound. The highest concentration of IAA was obtained from *Azotobacter* sp. which was designated as (A1).

The strain *Azotobacter* sp. (A1) was selected to characterize the production of IAA and the growth patterns as well as to investigate the effects of the pH and temperature on production of IAA.

Figure 2 illustrates that IAA formation started when the organism grew on the medium supplemented with tryptophan. The highest accumulation of IAA exhibited in the 48th hours of cultivation. The maximum accumulation of IAA occurred at the end of logarithmic phase, and after that the accumulation of IAA decreased at the beginning of the stationary growth phase. IAA accumulation coincided with increase in the specific growth rates of the cultures.

Effect of pH and temperature on production of indole-3-acetic acid by *Azotobacter* sp. (A1) isolate

Normally, the pH and temperature influence the growth of microorganisms and hence, these factors will influence also the production of IAA. The question is now, what are the optimal conditions (pH and temperature) for production of IAA by *Azotobacter* sp. (A1) isolate?

The influence of pH on biomass yield of the selected isolates is shown in Figure 3. As expected that the optimum pH was 7. The maximum of IAA yield production by *Azotobacter* sp. (A1) isolate was recorded at pH7. This bacterial isolate can grow at range from pH6-8.

The effect of different temperatures on production of IAA by *Azotobacter* sp. (A1) is shown in Figure 4. A temperature 30°C appears to be the optimal for production of IAA by the bacterial isolate. Therefore, the produced indole-3-acetic acid by this isolate at the optimum conditions (pH, temperature, at the end of logarithmic phase) was used for callus induction of *Dieffenbachia* cv. Marianne by using tissue culture technique rather than using synthetic IAA.

Our results are in agreement with previous findings reported by (Ernsten et al. 1987; Fukuhara et al. 1994; Glickmann and Dessaux 1995 and Torres-Rubio et al. 2000), who found that an addition of tryptophan in the growth medium led to production of IAA by many bacterial strains. Several different IAA biosynthetic pathways are used by prokaryotes, and a single bacterial strain can contain more than one pathway. Indole –3-acetic acid (IAA) biosynthetic pathways detected in *Erwinia herbicola pv.gypsophilae.* iaaM, iaaH, and ipdC are genes encoding tryptophan-2-monoxygenase, indole-3-acetamide hydrolase, and indole-3-pyruvate decarboxylase, respectively (Manulis et al. 1998).

Callus frequency percentage

Data presented in Table 2 and Figure 5 show that the difference among different growth regulators treatment were highly significant on callus frequency percentages. Explants grown on MS medium supplemented with 10 mg/l IAA + 5 mg/l BA gave the highest callus frequency percentage (97.22%) followed by explants grown on MS medium supplemented with 10mg/l bioauxin + 5 mg/l BA which produced (93.94%), while the control treatment (MS free medium) produced the fewest callus frequency percentage (34.52%). This due to

Table 2. Effect of growth regulators on callus induction and plantlets regeneration from *Dieffenbachia maculate* cv. Marianne.

Treatment	% Callus frequency	Callus fresh weight (g)	Callus diameter (cm)	Number of plantlet/callus
Free MS-medium (Control)	34.52 c	0.045	0.37 b	2.3 d
10 mg/l IAA	80.36 b	0.16	0.47 ab	7.5 c
5 mg/l BA	83.16 b	0.21	0.60 a	8.0 b
10 mg/l bioauxin	80.73 b	0.21	0.52 ab	8.5 b
10 mg/l IAA+ 5mg/l BA	97.22 a	0.14	0.59 a	10.67 a
10 mg/l bioauxin + 5 mg/l BA	93.94 ab	0.11	0.53 a	10.33 a
	**	n.s	**	**

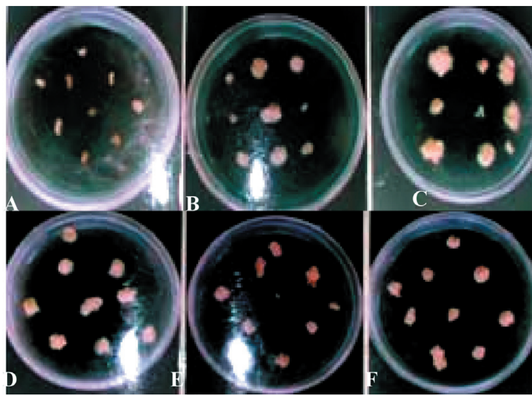

Figure 5. Callus formation of *D. maculata* cv. Marianne as affected by the different treatment after two months. A- control (MS free), B- MS with 10 mg/l IAA, C- MS with 5 mg/l BA, D- MS with10 mg/l bioauxin, E- MS with 10 mg/l IAA + 5 mg/l BA, F- MS with 10 mg/l bioauxin + 5 mg/l BA A.

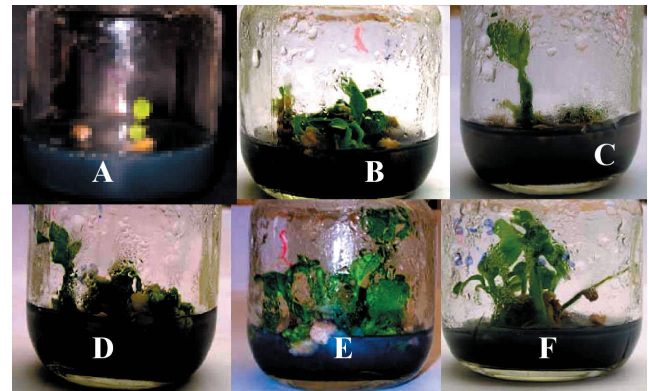

Figure 7. Regeneration plantlets of *D. maculata* cv. Marianne from callus on free -MS medium. A- control (MS free), B- MS with 10 mg/l IAA, C- MS with 5 mg/l BA, D- MS with10 mg/l bioauxin, E- MS with 10 mg/l IAA + 5 mg/l BA, F- MS with 10 mg/l bioauxin + 5 mg/l BA.

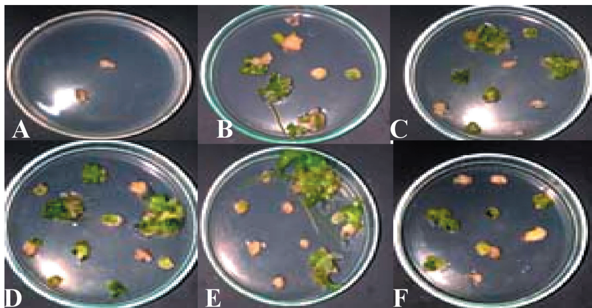

Figure 6. Plantlets formation of *D. maculata* cv. Marianne from callus on the differentiation medium. A- control (MS free), B- MS with 10 mg/l IAA, C- MS with 5mg/l BA, D- MS with10mg/l bioauxin, E- MS with 10 mg/l IAA + 5 mg/l BA, F- MS with 10 mg/l bioauxin + 5 mg/l BA A.

balance between auxin and cytokinin in the medium was nessacery for callus induction. It is evident that exogenous auxin is the most important growth regulator for the induction of embryogenic callus in the majority of angiosperms (Roy and Banerjee 2003). Also, as observed in the present study, *Dieffenbachia maculata* cv. Marianne requires supplementary cytokinin with auxin for optimum response of callus initiation. This requirement for exogenous cytokinin could be related to the maintenance of a proper balance between auxin and cytokinin, which acts synergistically to regulate cell division (Johri and Mitra 2001) a process essential for callus formation.

These results are in harmony with those obtained by Jain et al. (2002) on *Phlox paniculata* Linn., who found that, the intensity of callus proliferation was greater in the medium with BA in combination with IAA. Suthamathi et al. (2002) on papaya obtained the maximum callus induction from shoot tip with 10 mg/l NAA and 4.5 mg/l BA. Chamail et al. (1999) on *Actinidia deliciosa* found that, the best callus induction was obtained when BA (1.5 mg/l) was used in combination 1 mg/l NAA.

Callus fresh weight

Concerning the effect of growth regulators on the average callus fresh weight, there were no significant differences as shown in Table 2, but explants grown on MS medium supplemented with 5 mg/l BA or 10 mg/l bioauxin gave the highest callus fresh weight (0.21 g) followed by explants grown on MS medium supplemented with 10 mg/l IAA which produced 0.16 g for callus fresh weight. The variation might be due to endogenous levels of growth regulator (Kumar et al. 2001). The effect of growth regulator on tissue cultures can vary according to the chemical nature of the compound, plant species, type of the culture and even the developmental state of the explant (Lakshmanan et al. 2002). The obtained results were similar to those of Parthasarathy and Nagaraja (1999) on *Gerbera jamesonii* found that maximum callus weight was observed at 1.0 mg/l BAP.

Callus diameter

Regarding the effect of growth regulators on callus diameter, Data in Table 2 show that highly significant increase in callus diameter was recorded among the used treatments. Explants cultured on MS medium supplemented with 5 mg/l BA, 10 mg/l IAA + 5 mg/l BA and 10 mg/l bioauxin + 5 mg/l BA produced the biggest callus diameter with 0.60, 0.59 and 0.53 cm, respectively, while the control medium produced the smallest callus diameter 0.37 cm. Similar observation that the intensity of callus proliferation was greater in the medium with BA in combination with IAA for *Phlox paniculata* Linn and the response of callus production was more dependent on different concentration of BA and IAA than on the type

of explant source used (Jain et al. 2002). Induction of callus took place in the treatments that included NAA but the frequency was very low and the addition of BA was required for improved response (Roy and Banerjee 2003).

Number and plantlets/callus

Data in Table 2 and Figs (7 and 8) shown that there were highly significant differences in the number of plantlets/callus in differentiation medium (2 mg/l BA + 0.06 mg/l IBA). This may be due to the effect of induction medium. The largest number of plantlets/callus (10.67 and 10.33) resulted from callus was derived from induction medium consist of MS medium supplemented with 10 mg/l IAA + 5 mg/l BA and 10 mg/l bioauxin + 5 mg/l BA, respectively. In the present study, it was also observed that for callus differentiation, both of concentrations BA and IBA were essential to bring out the desirable change in callus differentiation rate. It is well known that for organogenesis, a proper balance between cytokinin and auxin is essential (Chamail et al. 1999). Similar results were reported by Chamail et al. (1999) on *Actinidia deliciosa* who found that 2 mg/l BA + 0.06 mg/l IBA was the best combination for producing maximum average number of shoots per callus.

In conclusion, induction of callus from shoot tips and internodal segments of *Dieffenbachia maculata* cv. Marianne occurred on different media but the presence of BA with auxin was essential for improved response. Also, the effect of bioauxin was similar to that of synthetic IAA on callus induction and regeneration.

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