Acta Bot. Croat. 60 (2), 157–168, 2001

CODEN: ABCRA 25
ISSN 0365–0588
UDC 581.085

Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Efficient shoot organogenesis of begonia (*Begonia rex* Putz.) induced by thidiazuron

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The effects of thidiazuron (TDZ) on adventitious shoot bud formation from leaf and petiole explants excised from in-vitro-grown plantlets of a begonia were tested by a two-step procedure: a short exposure (5 to 120 min) to 40 μM TDZ in liquid MS medium followed by culture on agar-solidified MS medium without growth regulators. Exposure of explants for five min to TDZ induced bud formation, but the number of buds was increased with prolonged exposure. Sixty min exposure to TDZ resulted in the highest mean number of regenerated shoots (24/leaf disc; 44/petiole segment after five weeks of culture). Shoots generated after two weeks in culture without, or with a small amount of, callus. During the third and fourth week in culture shoots developed small roots, and from the mother explant separated shoots elongated and further developed. The sucrose concentration in the basal expression medium influenced the efficiency of shoot organogenesis. The frequency of shoot formation tended to increase when the medium was supplemented with 2% sucrose instead with 3%.

Key words: *Begonia rex*, leaf disc, micropropagation, organogenesis, petiole segment, thidiazuron

Introduction

Over 200 species of begonias have been recorded, and they are primarily grown as ornamental plants (HUXLEY 1992). Still, this group of plants is also valued as a source of food and medical compounds. Begonias can be high in fructose, which determines that they may taste sweet or sour, and are used in the Philippines and Brazil as flavoring constituents. In Indonesia, China and Brazil begonia leaves are popular in salads (LAFERRIERE 1992). Certain species of begonia, including *Begonia gracilis*, are rich sources of biologically active phytochemicals (DOSKOTCH et al. 1968; LAFERRIERE 1992). Tubers of *B. gracilis* have been harvested to isolate cucurbitacins, which have been shown to have antitumor properties (DOSKOTCH and HUFFORD 1970), and roots have been used as purgatives, emetics, and cathartics (HERNANDEZ and GALLY-JORDA 1981; LAFERRIERE et al. 1991; LAFERRIERE 1992).

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Begonias are susceptible to many pathogenic bacteria, fungi, and nematodes. Interest in the use of cell culture techniques for the production of begonia plants has risen as a means of avoiding these hazards (TAKAYAMA 1990), and various micropropagation systems have been described. CHLYAH and TRAN THANH VAN (1975) report the morphogenetic capacity of *Begonia rex* Putz. (cv. President) explants composed of the epidermis and a few subjacent collenchyma layers. Buds are formed when zeatin or BA was added to the medium. FONNESBECH (1974) obtained shoot formation on petiole explants of *Begonia* x *cheimantha* grown on medium supplemented with BA. PECK and CUMMING (1984) used leaf sections as explants for micropropagation via organogenesis on solid medium. Liquid shake culture of *Begonia* x *hiemalis* was reported by SIMMONDS and WERRY (1987) as a means of inducing adventitious buds on petiole explants. The micropropagation of begonia using microshoots has limited scale-up potential because generally not more than ten cuttings can be harvested from a single proliferating shoot culture, and not more than six to ten divisions, each having a shoot primordium, can be obtained from a large tuber (PECK and CUMMING 1984). HVOSLEF-EIDE and SAEBO (1991) produced callus and cell suspensions of *Begonia* x *cheimantha* under red light irradiance, but found that, in darkness or under a broad spectrum of light wavelengths, explants were subjected to immediate organogenesis instead of sustained callus growth. CASTILLO and SMITH (1997) describe the induction of direct somatic embryogenesis on vegetative explants of *B. gracilis*.

Thidiazuron (TDZ) is a substituted phenylurea that is commercially used as a defoliant for cotton, and which also produces high cytokinin-like activity in in-vitro-cultivated cells (WANG et al. 1986; FIOLA et al. 1990; SAXENA et al. 1992). The mechanism of TDZ action is partly related to the inhibition of cytokinin degradation by cytokinin oxidase, resulting in increased levels of endogenous cytokinins (HARE and VAN STADEN 1994). Moreover, plant regeneration can be stimulated even though exposure to TDZ is for a relatively short time (VISSER et al. 1992). TDZ has been demonstrated to be very effective in inducing in vitro shoot regeneration of several species, such as kiwi fruit (SUEZAWA et al. 1988), apple (FASOLO et al. 1989), grape (MATSUTA and HIRABAYASHI 1989), pear (LEBLAY et al. 1991), peanut (KANYAND et al. 1994), Regal geranium (MURCH et al. 1997), eggplant (MAGIOLI et al. 1998), and several woody species (HUETTEMAN and PREECE 1993).

The present work was undertaken to determine the effect of TDZ on in vitro morphogenesis in *Begonia rex*. The effect of the duration of exposure to TDZ on bud induction, shoot elongation, and the subsequent development of plants was examined. In this paper we describe a procedure for high-frequency shoot induction from leaf and petiole tissues.

Materials and methods

Plant material

Leaf and petiole explants were excised from one-month-old in vitro shoots of *Begonia rex*. Donor cultures were maintained from 2 to 4-cm-long axillary shoots by routinely subculturing every 6–8 weeks to 300 ml Erlenmeyer flasks containing 50 ml of 1xMS medium (MURASHIGE and SKOOG 1962) supplemented with 3% sucrose and 0.8% agar.

Treatment with thidiazuron

Leaf discs (0.8 cm in diameter) each containing part of a vein, and petiole segments (0.8–1.0 cm) were treated with liquid 1/2xMS medium supplemented with MS vitamins, 2% sucrose, and 40 μM thidiazuron (TDZ, *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea) on a shaker for 0, 5, 15, 30, 60 and 120 min. Five treated explants were inoculated in a 90 x 15 mm disposable plastic petri dish containing ca. 25 ml 1xMS medium supplemented with MS vitamins, 0.9% plant agar and 2% or 3% sucrose. The medium was adjusted to pH 5.8 before autoclaving at 1.09 kg cm^{-2} for 20 min. The petri dishes were sealed with parafilm and incubated in a culture room. Petiole segments were placed horizontally on the medium's surface, and leaf discs were placed abaxial side down. Cultures were incubated at 24±1 °C a 16 h photoperiod, light 30–35 Wm^{-2}, provided by cool-white fluorescent tubes (Tež, Zagreb).

For further growth, separated, 0.5 to 1.5 cm long, shoots were transferred to 300 ml conical flasks, each containing 50 ml of MS basal medium without growth regulators, and placed in similar physical conditions in which they were induced.

Acclimatization of shoots

Separated shoots that had been cultured in 300 ml flasks for 4–5 weeks were taken out and gently washed with distilled water. They were then transplanted into plastic pots containing a wet mixture of perlite Agri, peat (Novobalt) and $CaCO_3$ (600 ml:400 ml:40 g). Plants were covered with polyethylene bags to avoid desiccation. Ronilan in a concentration of 0.1% was used against fungi. After 2 weeks, the polyethylene bags were completely removed.

Statistical analysis

Regenerated buds and shoots developed on the explants were counted weekly under a dissecting microscope. In the experiments, each treatment consisted of 15 experimental units and all experiments were repeated twice. The number of explants exhibiting regeneration was identified, and the number of shoots produced per explant determined. Values and means are followed by standard errors (± SE) of the means. The data on the number of shoots per explant were subjected to ANOVA and means were compared using Duncan's multiple range test at the 5% level of probability. A probability of $P \leq 0.05$ was considered significant.

Results

Leaf discs and petiole segments (30%–90%) formed shoot buds when exposed to 40 μM TDZ in liquid medium on a shaker and subsequently cultured on a solidified basal MS medium lacking growth regulators (expression medium). Short treatment with TDZ resulted in the formation of a normal shoot with one or two rootlets when it was still connected with the maternal explant. Regeneration occurred directly from the cells of the explants or from a small amount of callus formation (Figs. 1a, b).

Polarity in the explants was disrupted and buds could form on any part of a petiole segment or leaf tissue (Figs. 1b, c), but in the latter case the area on the main veins and cut edge

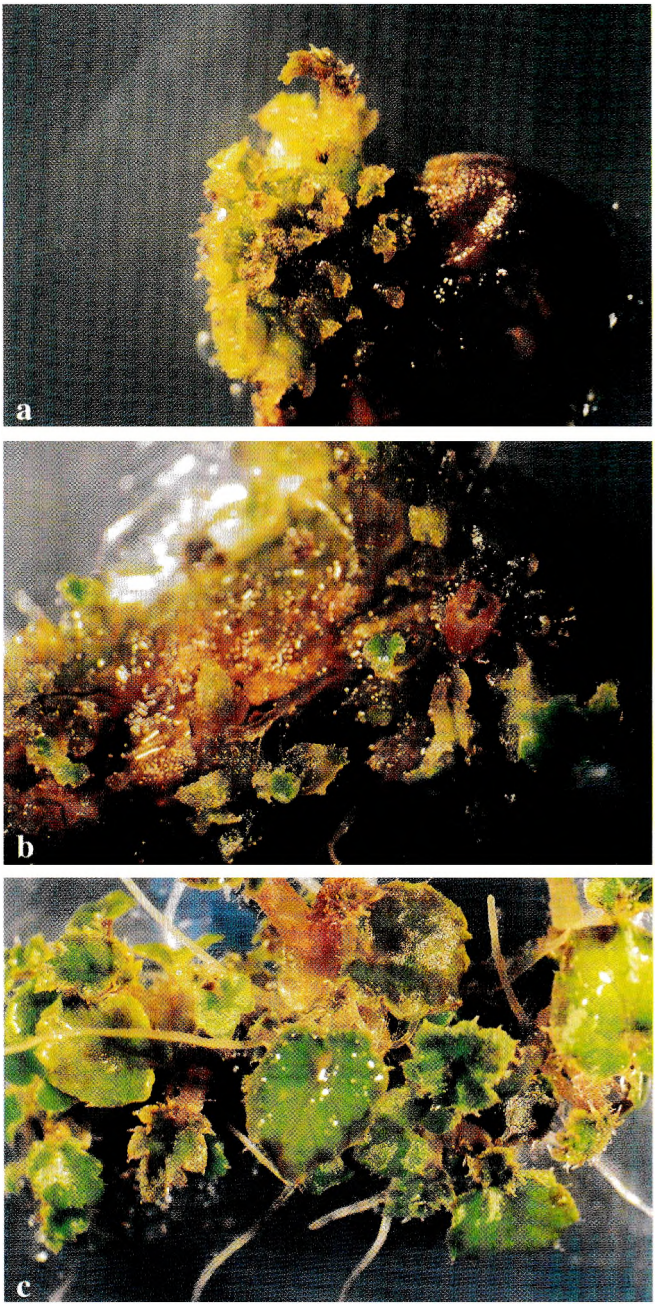

Fig. 1a-c. Adventitious shoot bud induction in explants of *Begonia rex* shortly exposed to 40 μM thidiazuron and cultured on a basal expression medium. (a) Cluster of adventitious buds developed on leaf disc exposed 5 min to TDZ. Photograph taken at 35 days after inoculation. (b) Many small shoots formed on petiole segment exposured 15 min to TDZ. Photograph taken at 35 days after inoculation. (c) Six-week-old leaf disc shows numerous well developed leaves, shoots and roots. The disc was exposed to TDZ for 15 min.

were most responsive. After one week of culture the petiole and leaf discs expanded and showed evidence of swelling at the cut end, and bud initials began to appear two weeks after culture initiation regardless of the duration of TDZ exposure, and continued to develop for 4–5 weeks and further (Tabs. 1–4). The regeneration capability varied between the two types of explants, petiole tissues showed a higher capacity for bud formation.

Length of TDZ treatment

The experiment performed to study the effect of the duration of exposure to TDZ revealed that the frequency of shoot bud production per explant mainly increased with increased duration of exposure to 40 μM TDZ (Tabs.1, 2). In the control treatment wherein the explants were transferred from TDZ-free treatment to expression medium, shoots were observed in a low number in petiole segments. They were sporadically produced from leaf discs only on the medium with 3% sucrose.

Tab. 1. Effect of the duration of exposure to 40 μM TDZ on the induction of shoots in *Begonia rex* leaf discs. Expression medium supplemented with 2% sucrose.

Duration of treatment min	Cultures with shoots %	Total no. of shoots	Mean number of shoots/reacted discs No. ± SE
0	0	0	0 ± 0 c
5	86	496	16.2 ± 2.3 b
15	50	603	20.1 ± 4.3 ab
30	90	554	18.1 ± 2.6 ab
60	83	722	24.1 ± 2.8 ab
120	80	810	27.0 ± 3.6 a

The means shoot number formed on each explant after 35 days of culture followed by different letters are significantly different at $P \leq 0.05$ (DMRT).

Tab. 2. Effect of the duration of exposure to 40 μM TDZ on the induction of shoots in *Begonia rex* leaf discs. Expression medium supplemented with 3% sucrose.

Duration of treatment min	Cultures with shoots %	Total no. of shoots	Mean number of shoots/reacted discs No. ± SE
0	16	13	0.4 ± 0.2 c
5	53	174	6.1 ± 1.2 b
15	36	199	6.6 ± 1.8 ab
30	26	194	6.5 ± 2.1 ab
60	60	344	11.5 ± 2.2 a
120	56	242	8.1 ± 1.5 ab

The means shoot number formed on each explant after 35 days of culture followed by different letters are significantly different at $P \leq 0.05$ (DMRT).

A 5 min treatment produced significantly higher numbers of shoots in leaf and petiole explants (mean 16 and 28/explant, respectively) than in the control (Tabs. 1, 3). The most effective treatment was 60 min, resulting in an average of 44 shoots per petiole segment (Tab. 3) and 24 shoots per leaf disc (Tab. 1).

Tab. 3. Effect of the duration of exposure to 40 μM TDZ on the induction of shoots in *Begonia rex* petiole segment. Expression medium supplemented with 2% sucrose.

Duration of treatment min	Cultures with shoots %	Total no. of shoots	Mean number of shoots/reacted discs No. ± SE
0	26	87	2.9 ± 0.9 c
5	83	848	28.3 ± 2.8 b
15	66	670	30.3 ± 1.7 b
30	66	711	29.1 ± 3.1 b
60	83	1129	43.6 ± 2.5 a
120	26	753	24.7 ± 4.1 b

The means shoot number formed on each explant after 35 days of culture followed by different letters are significantly different at $P \leq 0.05$ (DMRT).

Tab. 4. Effect of the duration of exposure to 40 μM TDZ on the induction of shoots in *Begonia rex* petiole segments. Expression medium supplemented with 3% sucrose.

Duration of treatment min	Cultures with shoots %	Total no. of shoots	Mean number of shoots/reacted discs No. ± SE
0	83	329	10.9 ± 1.2 b
5	66	653	21.8 ± 3.2 a
15	73	822	27.4 ± 2.8 a
30	66	695	23.1 ± 1.9 a
60	60	850	28.3 ± 4.4 a
120	83	798	26.6 ± 2.7 a

The means shoot number formed on each explant after 35 days of culture followed by different letters are significantly different at $P \leq 0.05$ (DMRT).

Effects of the sucrose concentration

The shoot induction was also affected by the concentration of sucrose (Tabs. 1–4). The number of induced shoots reached a maximum in an expression medium with 2% sucrose. Sucrose at 3% was somewhat superior to sucrose at 2% with respect to the number of shoots if petiole segments were exposed to TDZ longer than 60 min, and in the controls.

Shoot proliferation over an extended period

Shoot formation per explant increased during the 5 weeks in culture. Table 5 shows the dynamics of shoot formation in petiole explants. On the medium with 2% sucrose the high

Tab. 5. Dynamics of begonia shoot formation during the 5 weeks in culture expressed by the total number of shoots on the reacted petiole segments. Explants were treated with 40 μM TDZ. The expression medium contained 2% and 3% sucrose.

Duration of treatment/min	Week 1		Week 2		Week 3		Week 4		Week 5	
Sucrose	2%	3%	2%	3%	2%	3%	2%	3%	2%	3%
0	0	0	0	0	32	168	36	229	87	329
5	0	0	0	0	243	473	640	586	848	653
15	0	0	0	0	466	582	656	762	670	822
30	0	0	0	0	626	581	651	653	711	695
60	0	0	0	0	469	638	1101	719	1129	850
120	0	0	0	0	308	535	708	643	753	798

rate of bud multiplication continued over the entire five-week culture period and further (results are not shown), while on the medium with 3% sucrose more than half of the regenerated buds were formed in week 3.

Plant development

The shoot regenerants (1–1.5 cm) were separated from the maternal tissue and developed into mature plants on MS medium lacking growth regulators (Fig. 2a). We did not observe development of additional adventitious buds at the leaves on the shoots. The well developed plantlets were gently washed to remove agar and transplanted into pots containing a wet substratum. Plants were covered with polyethylene bags to maintain high humidity. After two weeks the bags were completely removed. The acclimatized plants showed normal growth and morphology (Fig. 2b).

Discussion

Earlier investigations on the regeneration of begonia species in tissue culture demonstrate that it is important for maximal shoot number and roots that growth regulators such as BA or zeatin alone, or BA and NAA in the appropriate ratio, are included in the medium (HEIDE 1965, RINGE and NITSCH 1968, FONNESBECH 1974, CHLYAH and TRAN THAN VAN 1975).

Our objective was to determine the effects of TDZ on in vitro regeneration of *Begonia rex* and, to our knowledge, this is the first time TDZ was tested for regeneration of *Begonia rex*.

Initial experiments (results are not shown) were performed to determine the effects of the concentrations and duration of exposure to TDZ on begonia explants. They revealed that transient exposure was sufficient to induce bud formation. We thus designed the experiments described in this article.

Bud regeneration in begonia explants was highly enhanced upon exposure to TDZ. In geranium, somatic embryogenesis can be achieved by maintaining hypocotyl explants for

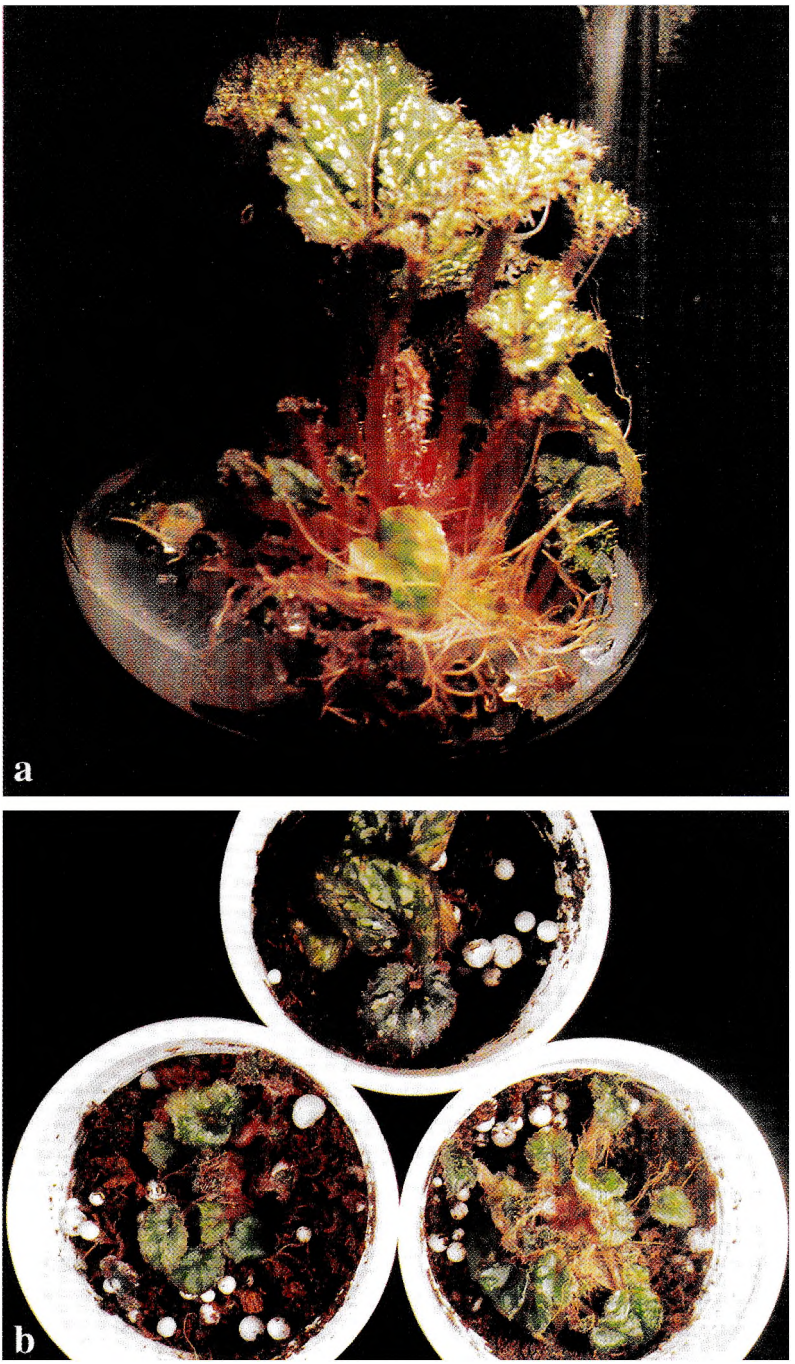

Fig. 2a, b. Well developed begonia plants obtained from explants exposured to TDZ. (a) Separated well-developed plantlet after 6 weeks in culture on 50 ml MS basal medium. (b) Plants transferred to sterile mixture of perlite, peat and $CaCO_3$, after two weeks acclimatization under a polyethylene bag, survived and became autotrophic.

3 days on an induction medium containing 10 μM TDZ and, subsequently, on basal medium (HUTCHINSON and SAXENA 1996). In our experiments not more than 5 min exposure to 40 μM TDZ induced a large number of shoots. Other authors show that the TDZ levels required were very low compared with other cytokinins (HUETTEMAN and PREECE 1993, KANEDA et al. 1997, FAURE et al. 1998, HOSOKAWA et al. 1998). Thidiazuron stimulated shoot formation in begonia without addition of auxin or other cytokinins to the medium. The effective use of TDZ as the sole plant growth regulator to induce regeneration has been reported for other species, as well (MALIK and SAXENA 1992, MURTHY and SAXENA 1998).

CHLYAH and TRAN THANH VAN (1975) investigated regeneration in *B. rex* explants composed of the epidermis and 4 or 5 subjacent collenchyma layers stripped from the main leaf veins. Buds were formed when benzyladenine or zeatin was added to the medium. They observed cell divisions at about the 8-day stage. After 12–14 days, a dome of newly formed cells was visible, and after 18–20 days, the meristem was well-structured and had formed the first foliar organ. In our experiments, bud formation had a similar chronology in both TDZ-treated cultures (regardless of the duration of the exposure) and in the controls.

The high frequency of shoot buds induced by TDZ suggests that it might influence the endogenous levels of hormones (cytokinins, auxins, abscisic acid) and stress-induced substances, so as to induce the positive organogenic response of the cultivated tissues. MURTHY et al. (1998) have reviewed the different potential modes of TDZ action.

Recently MURCH et al. (1997), on the base of their results attained on Regal geranium hypothesize that the primary effect of TDZ is through induction of a stress response in the plants. In order to overcome the stress, the plants accumulated significantly higher levels of proline, ABA and 4-aminobutyrate, and also exhibited modified metabolic processes which in turn led to increased availability of energy and reducing power required for subsequent growth and to initiate stress adaptation mechanisms including cellular processes and regenerative outgrowth development. SRIVANTANAKUL et al. (2000) also speculate that the regeneration plants is an adaptive reproduction mechanism of plants to overcome the stress induced by TDZ.

It is a general observation that in vitro morphogenesis depends on a variety of factors. Among others, explants, the medium regime, and growth regulators are important determining factors. The carbon source is one of the most important components for cell growth. Begonia explants treated with TDZ showed a decrease in shoot number with increasing sucrose concentrations which is in harmony with the data obtained in *Begonia* x *hiemalis* (TAKAYAMA and MISAWA 1981), *Lilium* (TAKAYAMA et al. 1991) and gentian (HOSOKAWA et al. 1998).

Petiole segments showed higher levels of regeneration efficiencies, as compared with leaf discs. That is consistent with observations on morphogenesis in *Pelargonium* x *hortorum* (AGARWAL and RANU 2000).

We did not notice necrosis of tissues, serious hyperhydricity, abnormal leaf morphology or inhibition of shoot elongation and root regeneration which were, in several papers, reported as TDZ effects (BRETAGNE et al. 1994, DEBERGH et al. 1992, van NIEUWKERK et al. 1986, HUETTEMAN and PREECE 1993). Subtle hyperhydricity that we observed after 60 and 120 min of TDZ exposure was reduced using a medium solidified with 0.9% agar.

Supplemental experiments involving additional concentrations, exposure times, combinations of several medium constituents could lead to greater precision in the use of TDZ for in vitro shoot production of begonia.

In conclusion, to propagate *Begonia rex* successfully, 1 cm long petiole (and leaf) segments could be cultured in 1xMS salts and vitamins medium supplemented with 2% sucrose and 0.9% agar without growth regulators, after a short TDZ exposure. The proposed procedure made it possible to obtain many rooted plantlets in a short time (less of two months), and has scale-up potential.

Acknowledgement

We thank Dr. Snježana Mihaljević for statistical assessment of the data, and Ms. Ana-Marija Boljkovac for excellent technical assistance. Ms. Ela Kosor is a graduate student. This research was partly supported by the Ministry of Science and Technology, Republic of Croatia, Projects No. 119113 and 119098.

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