Review of JPOP678

How is this an adventitious shoot regeneration protocol?

OPTIMIZATION OF HIGHLY EFFICIENT ADVENTITIOUS SHOOT FORMATION AND GROWTH OF *DROSERA INTERMEDIA* HAYNE – A POTENTIAL ORNAMENTAL PLANT

Jindrich Rejthar[a*], Iva Viehmannova[a], Petra Hlasna Cepkova[a]

[a]*Institute of Tropics and Subtropics. Czech University of Life Sciences Prague. Kamycka 129, Prague 165 21, Czech Republic*

(corresponding author; E-mail address: viehmann@its.czu.cz; Fax: +420 234381829)*

(E-mail addresses : rejtharj@its.czu.cz, cepkova@its.czu.cz)

Abstract

This study ~~is~~ aimed to optimize the *in vitro* propagation of *D. intermedia* for commercial and conservation purposes. The effect of nutrient concentration (four concentrations of MS nutrients), various pH (3.7-7.7), sucrose concentration (1-6% w/v) and cytokinins (0.1-3 mg l^{-1}), namely ~~BA~~ (N6-benzyladenine), kinetin and zeatin was evaluated. After 60 days of seedling cultivation, growth and developmental characteristics were recorded. No significant differences were found for various levels of pH and sucrose. ~~On the contrary,~~ plant height was negatively influenced by an increase of nutrients in the medium. The plants on 1/8 MS medium were significantly ~~higher~~, and displayed more intensive proliferation capacity compared with those ~~cultivated~~ on ~~the~~ MS medium. Shoot multiplication and growth was suppressed by supplementation of BA and kinetin, regardless of concentration used. Zeatin at the lowest concentration (0.1 mg l^{-1}) provided the best results for shoot proliferation of all 26 media tested and can be recommended for micropropagation of *D. intermedia*.

State the complete zeatin medium here (strength of MS, sucrose, zeatin, agar, pH).

Key words: Droseraceae; *Drosera intermedia*; micropropagation; oblong-leaved sundew; zeatin

* Report all plant growth regulators in micromolar (µM) not milligrams per liter. *

1. Introduction

The *Drosera* genus (Droseraceae) consists of carnivorous plants with active flypaper traps and includes nearly 150 species distributed in Australia, Africa, and South America, with some Northern Hemisphere species (Rivadavia et al. 2003). Some species, mainly *Drosera rotundifolia* L., *D. intermedia* Hayne and *D. anglica* Huds., have been commonly used as a traditional medicine in the therapy of respiratory tract (Paper et al. 2005, Fukushima et al. 2009). ~~Recently,~~ several bioactive compounds from sundew leaves and roots including flavonoids and quinones have been found (Marczak et al. 2005, Hook 2001, Putalun et al. 2010). In addition to the use for pharmaceutical applications (Biteau et al. 2012), *Drosera* species have long held the interest of botanists and horticulturists because of their unique biology and carnivorous habit (Wolf et al. 2006, Kawiak et el. 2003), and today, the most important economic use is for the ornamental plants trade (Barthlott et al. 2004).

spell out

D. intermedia is an attractive species of potential horticultural value. It is sized between *D. anglica* and *D. rotundifolia*, bearing wedge-shaped leaves, 7-12 mm in length, ~~long~~, and 4-10 mm wide, in a rosette. All the tentacles on the leaf surface are of symmetric length. Inflorescences are cymose or racemose, more robust than those of *D. rotundifolia*, composed of white flowers (Crowder et al. 1990, Wynne 1944). *D. intermedia* ~~had~~ has been used for artificial hybridization with *D. anglica* and *D. capillaris* (Kusakebe 1979). Subesquently, a spontaneous hybrid *D. intermedia* x *D. capillaris*, interspread between colonies of the two species, was discovered (Sharidan 1987).

spell out

check spelling with Reference

In nature, *D. intermedia* grows in the acidic parts of valley mires that are flooded in winter, and subject to drying out in summer, but it is also widespread in persistent pools. In most natural sites of Europe and North America, it is becoming less frequent because of land

drainage and uncontrolled collections for medicinal and ornamental purposes (Crowder et al. 1990, Kawiak et al. 2003, Kawiak et al. 2011). Therefore, *in vitro* propagation would be a useful tool for conservation of germplasm and as a source of plants for commercialization of the species (Jimenez et al. 2011, Swart et al. 2012).

In *Drosera* genus, micropropagation has been successfully optimized for *D. indica* (Jayaram and Prasad 2007), *D. aliciae* (Kawiak et al. 2100), *D. peltata* (Kwang-Soo and Go-Won 2004), *D. rotundifolia* (Bobak et al. 1995), *D. capensis* (Jiménez et al. 2011), *D. spatulata* (Bobak et al. 1993, Perica and Berljak 1996), and *D. anglica*, *D. binata*, and *D. cuneifolia* (Kawiak et al. 2003).

Until now, there was just one brief report on *in vitro* micropropagation of *D. intermedia* (Grevenstuk et al. 2010). Thus, the aim of this study was to establish an efficient micropropagation protocol for this vulnerable and attractive species, extensively testing responses to different abiotic factors, especially the concentration of mineral salts, plant growth regulators and sucrose in cultivation media, as well as various pH of the media.

2. Materials and methods

2.1. Plant material and establishment of *in vitro* culture

As the initial plant material for the experiment, the seeds of *Drosera intermedia* (Droseraceae) were used. They were obtained via *Index Seminum* from Universitatea Babeş-Bolyai, Gradina botanica-Al Borza, Cluj-Napoca, Romania, in the year 2009. The seeds were disinfected in 70% ethanol for 1 min, followed by 20-25 minutes in 2% NaClO containing a few drops of the detergent Tween 20. After sterilization, the plant material was rinsed three times for one minute in sterile, distilled water, and placed on half-strength MS medium

(Murashige & Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 100 mg l^{-1} *myo*-inositol and pH adjusted to 5.7. Cultures were maintained at 25/23°C under a 16/8 h light/dark regime with 36 µmol m^{-2} s^{-1} cool white fluorescent light (NARVA LT 36 W/010). The seedlings (ca 5 mm in height) obtained from the seeds after 30 days ~~of cultivation~~ were used for multiplication experiment.

2.2. *In vitro* multiplication of plants

For multiplication, in total 25 treatments were tested. All media were derived from basal medium, consisting of half-strength MS medium ~~(Murashige & Skoog, 1962)~~ supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 100 mg l^{-1} *myo*-inositol and pH adjusted to 5.7. This medium was also used as control.

In order to investigate optimal concentration of sucrose for multiplication, media containing 1%, 2%, 3%, 4%, 5%, ~~and~~ 6% (w/v) were tested. The effect of nutrient concentration was investigated using full-strength MS, 1/2 MS, 1/4 MS, ~~and~~ 1/8 MS ~~media~~ ~~in the experiment.~~ To detect the influence of various pH, the media pH was adjusted to 3.7, 4.7, 5.7, 6.7 ~~and~~ 7.7 (KOH, citric acid) prior to autoclaving. The cytokinin effect was tested ~~on~~ ~~media~~ enriched ~~by cytokinins, namely~~ kinetin, N6-benzyladenine (BA), ~~and~~ zeatin at four concentrations (0.1, 0.5, 1, 3 mg l^{-1}).

Cultures were maintained under the cultivation conditions described ~~above~~. After 60 days of ~~cultivation~~, ~~plant~~ height, the number of shoots per plant, diameter of rosettes, and the number and length of roots were recorded.

2.3. Experimental design and statistical analysis

All experiments were repeated twice with 15 replications per each treatment. The experiment was arranged in a completely randomized block design. The data were subjected to analysis of variance (ANOVA) and the least significant ($p < 0.05$) differences among mean values were estimated using Fisher's LSD test [StatSoft STATISTICA 9.0].

need to cite A Reference for this

2.4 *Ex vitro* transfer and acclimatization

How were shoots rooted?

Well rooted plants were rinsed with tap water to remove the adhering medium and then planted separately in pots filled with a mixture of peat moss and perlite 1:1 (v/v). The plants were grown under high air humidity (85%) for 7 days. For the next 14 days, relative humidity was reduced and light intensity was gradually increased. The success of acclimatization was determined after 8 weeks as the survival rate.

3. Results

Use cultured instead of cultivated.

3.1 Multiplication

Seedlings? Shoots?

~~The results revealed that~~ plants cultivated on medium with lower concentrations of sucrose (1%, 2%) showed higher proliferation capacity and had a higher diameter of rosettes, although the differences among these characteristics were not statistically significant when compared to other treatments. ~~Moreover,~~ these plants were rather tiny and displayed the tendency to produce flowers under *in vitro* conditions (data not shown). ~~On the contrary,~~ a treatment containing 4% sucrose produced thick lower plants, with smaller diameter of rosettes, and in some individuals of abnormal morphology. In all treatments, plant height, number of roots per plant and ~~their~~ root length were comparable (Table 1).

The effect of nutrient concentration on measured characteristics after 60 day cultivation is shown in Table 1. It is evident, that plant height was negatively influenced by an increase of nutrients in the medium. The plants on 1/8 MS medium were significantly higher compared to those cultivated on MS medium. In addition, they displayed high proliferation capacity, though the statistical differences were not confirmed for this characteristic. Reducing the concentration of nutrients did not have any effect on diameter of rosettes and number of roots. On the contrary, the roots of plants cultivated on media with decreased concentrations of nutrients were singnificantly longer and thinner in comparison to those of plants grown on full-strength medium (data for root thickness are not shown).

When the effect of different pH values on growth and development characteristics was investigated, almost no significant differences among treatments were observed (Table 1). Overall, media with lower pH level (3.7, 4.7) produced tiny plants, while individuals cultivated on media with higher values of pH (6.7, 7.7) were rather thick and robust. These differences, however, did not have any impact on success of *ex vitro* transfer and acclimatization of plants.

Plant growth and development was strongly affected by cytokinins used. Homogeneous behaviour was observed for the cultures on media, supplemented with BA and kinetin at all concentrations (Table 1). These treatments produced extremely dwarf plants, morphologically abnormal (Fig. 1A). Moreover, the plants did not display any shoot and root production. Red pigmentation and necrosis in these cultures abundantly occurred. Of these treatments, only 3 mg l^{-1} BA provided some multiplication and root formation (Table 1). The plants, however, were even smaller compared to the other treatments, and the necrosis was more frequent in these cultures.

In contrast, zeatin proved to be optimal for *D. intermedia* propagation. This treatment provided the highest shoot proliferation of all 26 media tested (Fig. 1B). Also the diameter of

rosettes and length of roots were significantly higher when compared to other cytokinin treatments. The increasing concentration of zeatin suppressed the growth and development of cultures.

3.2. *Ex vitro* transfer and acclimatization

Shoot clusters developed on media were divided into separate shoots and transferred *ex vitro*. Well rooted plants were used for *ex vitro* transfer, survival rate after 8 weeks was 98.6%. Plants displayed ~~vital and~~ vigorous growth, ~~A~~any abnormalities observed *in vitro* gradually disappeared during further 4 weeks.

4. Discussion

In the present work, a protocol for ~~effective~~ *in vitro* propagation of *Drosera intermedia* has been elaborated, testing a wide range of ~~cultivation~~ media. This is the first complex study on multiplication of this species, extending the previous research of Grevenstuk et al. (2010).

Since *Drosera* plants grow normally in nutrient poor habitats (Jayaram and Prasad 2007), different strengths of MS medium ~~had been~~ tested. The superiority of 1/8 MS medium over other concentration of MS medium was shown, although 1/4 MS and 1/2 MS media also provided satisfactory results. Similarly, Grevenstuk et al. (2010) recommended for multiplication of the same species 1/4 MS medium, ~~the least concentrated medium,~~ which ~~had been~~ tested in ~~the~~ study. Unlike our results, in *D. spatulata*, shoot proliferation was significantly decreased on diluted MS medium (Perica and Berljak 1996), and in *D. indica*, the plant multiplication was not markedly influenced by different nutrient concentration (Jayaram and Prasad 2007).

Surprisingly, in our experiment no significant differences were detected among different pH (3.7-7.7). Since sundews grow in natural sites on acid soils, where pH ranges between 3.5 and 4.7 (Juniper et al. 1989, Crowder et al. 1990), markedly better results for multiplication had been expected under lower pH. These data do not correspond with those of Kwang-Soo and Go-Won (2004) for *D. peltata*, where significantly higher shoot proliferation was achieved at pH 5.7, while pH 3.7 substantially decreased this characteristic.

For *in vitro* cultures, the main source of carbohydrates, providing energy for growth and biosynthetic processes, is sucrose (de Melo Ferreira et al. 2011). Thus, the effect of its concentration on growth and developmental characteristics was also examined. No statistical significant differences between concentrations, however, were detected. These findings confirm those of Jayaram and Prasad (2007) for *D. indica*, where three sucrose concentrations provided very similar results. Jimenez et al. (2011) in *D. capensis* suggested that the highest increase in fresh weight can be achieved by low concentration of sucrose, but the optimal level of sucrose may depend upon other components of the culture medium, such as mineral salts and organic substances.

The inductive effect of cytokinins on morphological characteristics had been tested in our study. Synthetic cytokinins, i.e. BA and kinetin were not suitable as they did not allow good shoot proliferation when compared to the effect of zeatin. In addition, BA and kinetin supplementations caused extreme suppression of growth and hyperhydricity of *in vitro* tissues. Grevenstuk et al. (2010), however, did not report in the study on *D. intermedia,* a negative effect of kinetin on shoot multiplication when compared to control medium without plant growth regulators. Likewise, BA alone or in combination with NAA provided high micropropagation coefficient in *D. aliciae*, *D. anglica* and *D. cuneifolia* (Kawiak et al. 2003, Kawiak et al. 2011).

In our experiment, zeatin proved to be the most suitable cytokinin for multiplication of *D. intermedia*. The lowest ~~tested~~ concentration used (0.1 mg l^{-1} zeatin) provided the highest number of shoots per plant from all 26 ~~tested media~~. Superior effect of zeatin over kinetin and BA had been reported also for micropropagation of other species (Peixe at al. 2007, Hendrawati et al. 2012). Although zeatin is very expensive ~~chemical compound~~, it is the only growth regulator capable of inducing satisfactory growth and multiplication, and thus it is not possible to replace it by another cytikinin.

Acclimatization to greenhouse conditions was successfully achieved in most plants (98.6%), which may be attributed to the fact that for *ex vitro* transfer were used only well developed and rooted plants. ~~Furthermore~~, a mixture of peat moss and perlite represents ~~the optimal~~ substrate for acclimatization and continuous growth of plants, as it allows a certain degree of water retention, and permits good drainage and aeration of roots (Jimenez et al. 2011).

To summarize, various ~~levels of abiotic~~ factors may greatly affect growth and development of *in vitro* cultivated plants of *D. intermedia*. A decreased concentration of nutrients in ~~cultivation~~ medium and supplementation of zeatin at low concentrations seems to be adequate for micropropagation of the species, while different pH values and sucrose concentrations do not influence the multiplication rate significantly. Based on these results, ~~the optimized~~ protocol can be used for large scale clonal propagation of the species for commercial and conservation purposes.

Acknowledgments

This research was financially supported by Internal Grant Agency of Institute of Tropics and Subtropics, Czech University of Life Sciences Prague (Project No. 51110/1312/3115).

References

Check all References for accuracy ✓

Barthlott, W., Porembski, S., Seine, R., Theisen, I. (2004). Karnivoren: Biologie und Kultur Fleischfressender Pflanzen. Ulmer Eugen Verlag, Stuttgart, 224 pp.

Biteau, F., Nisse E., Alain H., Miguel, S., Hannewald P., Bourgaud F. (2012). A rapid and efficient method for isolating high quality DNA from leaves of carnivorous plants from the *Drosera* genus. Molecular Biotechnology, 51: 247–253.

Bobak M., Blehova A., Kristin, J., Ovecka, M., Samaj, J. (1995). Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*. Plant Cell Tissue and Organ Culture, 43: 43–49.

Bobak M., Blehova A., Kristin, J., Ovecka, M., Samaj, J. (1993). Studies of organogenesis from the callus culture of the sundew (*Drosera spathulata* Labill.). Journal of Plant Physiology, 142: 251-253.

Crowder, A.A., Pearson, M.C., Grubb, P.J., Langlois, P.H. (1990). Biological flora of the British Isles: *Drosera* genus L. Journal of Ecology, 78: 233–267.

Ferreira, W. de Melo, Suzuki, R.M., Pescador, R. (2011). Propagation, growth, and carbohydrates of *Dendrobium* Second Love (Orchidaceae) *in vitro* as affected by sucrose, light, and dark. In Vitro Cellular & Developmental Biology-Plant, 47: 420–427.

Fukushima, K., Nagai, K., Hoshia, Y., Masumoto, S., Mikami I., Takahashib, Y., Oikeb, H., Kobori, M. (2009). *Drosera rotundifolia* and *Drosera tokaiensis* suppress the activation of HMC-1 human mast cells. Journal of Ethnopharmacology, 125: 90–96.

Grevenstuk, T., Coelho, N., Gonçalves, S., Romano, A. (2010). *In vitro* propagation of *Drosera intermedia* in a single step. Biologia Plantarum, 54: 391-394.

Hendrawati, O., Hille, J., Woerdenbag, H. J., Quax, W.J., Kayser, O. (2012). *In vitro* regeneration of wild chervil (*Anthriscus sylvestris* L.). In Vitro Cellular & Developmental Biology-Plant, 48: 355–361.

Hook, I.L.I. (2001). Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera* species. Plant Cell Tissue and Organ Culture, 67: 281–285.

Jayaram, K., Prasad, M.N.V. (2007). Rapid *in vitro* multiplication of *Drosera indica* L.: a vulnerable, medicinally important insectivorous plant. Plant Biotechnology Reports, 1: 79–84.

Jiménez, V.M., Guevara, E., Masis E. (2011). Effect of macronutrients and sucrose concentration on *in vitro* grow of *Drosera capensis L.* (Droseraceae) plants, and evaluation of six substrates for aclimatization. Propagation of Ornamental Plants, 5: 47– 68.

Juniper, B.E., Robins, R.J., Joel, D.M. (1989). The Carnivorous Plants. Academic Press, London, 353 pp.

Kawiak, A., Lojkowska, E. (2011). *In vitro* cultures of *Drosera aliciae* as a source of a cytotoxic naphthoquinone: ramentaceone. Biotechnology Letters, 33: 2309-2316.

Kawiak, A., Królicka, A., Lojkowska, W. (2003). Direct regeneration of *Drosera* from leaf explants and shoot tips. Plant Cell Tissue and Organ Culture, 75: 175–178.

Kusakabe, I. (1979). Japanese *Drosera* hybrids. Carnivorous Plant Newsletter, 8: 54.

Kwang-Soo, K., Go-Won, J. (2004). Micropropagation of *Drosera peltata*, a tuberous sundew, by shoot tip culture. Plant Cell Tissue and Organ Culture, 77: 211–214.

Marczak, L., Kawiak, A., Lojkowska, E., Stobiecki, M. (2005). Secondary metabolites in *in vitro* cultured plants of the genus *Drosera*. Phytochemical Analysis, 16: 143–149.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 474–497.

Paper, D.H., Karall, E., Kremser, M., Krenn, L. (2005). Comparison of the antiinflammatory effects of *Drosera rotundifolia* and *Drosera madagascariensis* in the HET-CAM assay. Phytotherapy Research, 19: 323–326.

Peixe, A., Raposo, A., Lourenço, R., Cardoso, H., Macedo, E. (2007). Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. Scientia Horticulturae, 113: 1-7.

Perica, M.C., Berljak, J. (1996). *In vitro* growth and regeneration of *Drosera spatulata* Labill on various media. Hortscience, 31: 1033–1034.

Putalun, W., Udomsin, O., Yusakul, G., Juengwatanatrakul, T., Sakamoto, S., Tanaka, H. (2010). Enhanced plumbagin production from *in vitro* cultures of *Drosera burmanii* using elicitation. Biotechnology Letters, 32: 721–724.

Rivadavia, F., Kondo, K., Kato, M., Hasebe, M. (2003). Phylogeny of the sundews, *Drosera* (Droseraceae) based on chloroplast rbcL and nuclear 18S ribosomal DNA sequences. American Journal of Botany, 90: 123–130.

Sheridan, P. (1987). A Preliminary Report on *Drosera intermedia* x *D. capillaris*. Carnivorous Plant Newsletter, 16: 71-73.

Swart, P.A., Kulkarni, M.G., Bairu, M.W., Finnie, J.F., Van Staden, J. (2012). Micropropagation of *Romulea sabulosa* Schltr. ex Beg. - A potential ornamental plant. Scientia Horticulturae, 135: 151–156.

Wolf, E., Gage, E., Cooper, D. (2006). *Drosera rotundifolia* L. (roundleaf sundew): A technical conservation assessment. Report prepared for the USDA Forest Service, Rocky Mountain Region, Species Conservation Project. Available online at: http://www.fs.fed.us/r2/projects/scp/assessments/droserarotundifolia.pdf.

Wynne, F.E. (1944). *Drosera* in eastern North America. Bulletin of the Torrey Botanical Club, 71: 166-174.

Table

Tab. 1: Effect of nutrient concentration, pH, sucrose level and cytokinins on growth and developmental characteristics in *Drosera intermedia*

Tested treatments	Plant height (mm)	No of shoots/explant	Diameter of rosette (mm)	No of roots/explant	Length of roots (mm)
Nutrient concentration					
1/8 MS	24.34±3.60ef	4.27±1.32ab	20.16±0.04cd	6.67±1.03abc	37.50±2.74efg
¼ MS	21.35±3.32def	2.18±1.07ab	27.52±2.74d	6.63±1.39abc	39.13±4.05fg
½ MS	20.10±4.46def	2.60±2.08ab	26.03±8.28d	8.67±2.41abc	37.51±2.72efg
MS	12.30±2.24bcd	3.17±1.83ab	17.72±4.74bcd	11.57±2.71c	25.73±4.66bcd
pH					
½ MS pH 3.7	15.22±3.85cde	2.17±1.47ab	22.89±5.27cd	5.65±3.47abc	39.23±4.03fg
½ MS pH 4.7	17.47±4.17def	2.51±2.36ab	26.67±7.63d	6.77±5.82abc	46.57±5.17g
½ MS pH 5.7	20.03±5.21def	2.48±2.17ab	25.13±7.88d	8.97±2.28abc	36.48±2.54efg
½ MS pH 6.7	14.82±4.73cde	2.44±1.75ab	21.47±2.54cd	4.08±1.89ab	34.07±4.90def
½ MS pH 7.7	15.20±3.70cde	3.13±2.16ab	23.13±4.28cd	9.51±5.68abc	28.29±8.16cde
Sucrose concentration					
½ MS 1% sucrose	25.27±9.43f	4.13±2.16ab	26.56±4.58d	11.50±4.10c	21.33±9.88bc
½ MS 2% sucrose	18.33±3.98def	4.32±2.75ab	25.93±7.72d	9.46± 4.93abc	28.33±4.06cde
½ MS 3% sucrose	19.02±4.44def	3.16±2.04ab	25.41±8.35d	9.67±3.34abc	37.63±2.54efg
½ MS 4% sucrose	17.91±6.43def	3.33±2.42ab	20.85±8.07cd	10.51±4.48bc	25.04±4.45bcd
Cytokinins					
0	19.51±4.37def	2.53±2.10ab	26.03±7.37d	8.58±2.32abc	36.99±2.64efg
Kinetin (mg l^{-1})					
0.1	4.79±0.43ab	1.00±0.00a	5.32±1.19ab	0.00±0.00	0.00±0.00
0.5	4.50±2.27ab	1.00±0.00a	4.87±0.42ab	0.00±0.00	0.00±0.00
1	4.18±0.96ab	1.00±0.00a	5.03±1.27ab	0.00±0.00	0.00±0.00
3	4.02±1.24ab	1.00±0.00a	4.66±1.03ab	0.00±0.00	0.00±0.00
BAP (mg l^{-1})					
0.1	6.19±2.08abc	1.00±0.00a	4.42±1.51ab	0.00±0.00	0.00±0.00
0.5	6.28±2.12abc	1.00±0.00a	3.67±1.52ab	0.00±0.00	0.00±0.00
1	4.99±0.89ab	1.00±0.00a	3.33±1.05ab	0.00±0.00	0.00±0.00
3	1.96±1.45a	2.32±1.51ab	1.75±1.30a	2.57±2.06a	2.33±2.06a
Zeatin (mg l^{-1})					
0.1	25.92±3.66f	10.51±2.50c	41.34±4.12e	8.64±3.68abc	18.31±2.41b
0.5	17.27±8.68def	5.17±1.17b	27.17±11.52d	5.84±1.57abc	6.17±2.86a
1	13.19±5.76bcd	4.54±2.27ab	19.67±9.27cd	2.67±1.96a	6.00±2.90a
3	5.85±2.14abc	2.65±0.53ab	10.82±2.06abc	0.00±0.00	0.00±0.00

[a] Mean values in column followed by the different letters ~~are~~ were significantly different according to the Fisher's LSD test ($p < 0.05$).

[b] Data were recorded after 60 days of culture.

306 medium with 1 mg l⁻¹ kinetin; bar = 1 mm. (B) Shoot multiplication on ½ MS with 0.1 mg l⁻¹

307 zeatin after 60 days of cultivation; bar = 10 mm.

308

How were shoots rooted?

It would be good to show AN acclimatized plant in the greenhouse or culture room.

It is not clear to me how this is AN Adventitious shoot regeneration protocol. Were seeds basically germinated in vitro And then these in vitro seedlings placed on various treatments? This needs to be clarified in the text. This would make it AN Axillary shoot production OR shoot proliferation protocol.

A