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BRIEF COMMUNICATION

Effect of vessel type and growth regulators on micropropagation of *Capsicum annuum*

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Abstract

Leaves from 14-d-old *Capsicum annuum* L. cv. Anaheim seedlings were cultured on Murashige and Skoog (MS) medium containing different combinations of indole-3-acetic acid (IAA) and 6-benzyladenine (BA). After 3 months, cultures were transferred to new medium where BA was replaced with 9 μM isopentenyladenine (2iP) to enhance the growth of shoot buds. Developing shoots were elongated and rooted on MS medium enriched with 9 μM indole-3-butyric acid (IBA). All cultures were maintained in 250 cm^3 baby jars covered with a clear polypropylene lid with or without microporous polypropylene membrane. Vessel type and plant growth regulators significantly affected callus morphogenic appearance, organogenesis and *in vitro* plantlet growth. Ventilated vessels supported photomixotrophic culture and improved regeneration and growth of plantlets. Higher plantlet dry mass and content of photosynthetic pigments, and lower stomatal density of plantlets grown in ventilated than in non-ventilated vessels facilitated *ex vitro* acclimation and growth.

Additional key words: callus, chlorophylls, *in vitro* growth, pepper, regeneration, stomata, total soluble sugar content, ventilation.

Pepper (*Capsicum* spp.) is an economically important vegetable and hot spice crop as its fruits account about 22 % of the total global spice trade. It is source for colours and antioxidant. The pungency of red pepper fruits is caused by capsaicinoids, which are used in the pharmaceutical industry (Szallasi 2002).

Sufficient ventilation of vessels using for micropropagation, which is dependent on their closure type, allows gas exchange preventing the significant accumulation of ethylene and depletion of CO_2. These conditions increase the photosynthetic capacity, the multiplication rate, and the survival during acclimatization (Hazarika 2006). An effect of CO_2 transported through microporous closures (ventilation) on the amount of photosynthetic pigments in tobacco plants has been reported. Moreover, ventilation increased net photosynthetic rate, and lowered transpiration rate and stomatal conductance under *ex vitro* conditions (Haisel *et al*. 1999). High vessel humidity causes anatomical abnormalities which may lead to high mortality during *ex vitro* transfer. Therefore, special closures that facilitate water loss could reduce the relative humidity inside the vessel and consequently improve plantlet growth under greenhouse conditions (Hazarika 2006).

Although, some members of family *Solanaceae* can easily undergo morphogenesis, red pepper was found to be recalcitrant. Nevertheless, few protocols for its micropropagation have been reported. These reports suggest a strong influence of genotype on micropropagation and a low regeneration rate of many cultivars including Anaheim (see Kothari *et al*. 2010). Ochoa-Alejo and Ireta-Moreno (1990) achieved only 1.12 - 1.61

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Abbreviation: BA - 6-benzyladenine; DM - dry mass; FM - fresh mass; 2iP - isopentenyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; SEM - shoot elongation medium; SIM - shoot induction medium; VV - ventilated vessels; NVV - non-ventilated vessels.
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shoots per explant of *C. annuum* cv. Anaheim on Murashige and Skoog (1962; MS) medium enriched with various combinations of growth regulators. Therefore, the aim of this study was to investigate the effect of vessel type and plant growth regulators on the regeneration efficiency of this species and improve its micro-propagation protocol.

Seeds of *Capsicum annuum* L. cv. Anaheim (obtained from *Bursa Seed*, Bursa, Turkey) were surface sterilized with 10 % commercial bleach for 15 min and washed several times with sterile water. They were incubated for 4 d on MS medium under dark at temperature 22 ± 1 ºC. Germinated seeds were transferred to 16-h photoperiod with irradiance of 100 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. After two weeks of incubation, the cotyledons were dissected, their tops and bases gently removed, and these explants were cultured on shoot induction medium (SIM) consisted of MS medium supplemented with 0, 11 and 22 µM indole-3-acetic acid (IAA) + 12, 24 and 36 µM 6-benzyladenine (BA) + 30 g dm^{-3} sucrose. The medium was distributed into 250 cm^3 baby jars and closed with polypropylene rigid closure with (VV) or without (NVV) vents (microporous polypropylene membrane, 0.22 µm pore size; *Sigma*, St. Louis, USA). For each treatment there were 10 baby jars replicates each with 5 explants.

Cultures were transferred onto fresh medium 3 times at monthly intervals. To enhance shoot elongation, explants were transferred twice at monthly intervals into shoot elongation medium (SEM) which was MS medium containing the same concentrations of IAA as in SIM and 9 µM isopentenyladenine (2iP). Developing shoots (> 2 cm) were separated and cultured in the same type of vessels as in the previous stages using MS medium supplemented with 20 g dm^{-3} sucrose and 9 µM indole-3-butyric acid (IBA) for rooting. All cultures were incubated under the same conditions as mentioned for seedling growth, media were solidified with 8 g dm^{-3} agar (*BDH Laboratory Suppliers*, Poole, UK) and the pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min.

After 2 months on rooting medium, 9 randomly rooted plantlets were selected from each treatment to measure shoot and root length and fresh mass (FM) of plantlets before drying at 70 °C to estimate their dry mass (DM). Total soluble sugars (TSS) were assessed using 4 samples (each 100 mg DM) as described by Jermyn (1975). The rest of plantlets were transferred into the greenhouse. Before transplanting into the greenhouse, 4 samples (100 mg) from the middle part of full expanded leaves were collected and photosynthetic pigments were extracted using dimethyl sulphoxide to measure chlorophyll *a, b* and total chlorophyll contents (Richardson *et al.* 2002). After one month of acclimatization, plantlet FM, DM, TSS and chlorophyll contents were estimated from 9 fully expanded newly developed leaves randomly detached. Stomatal density was estimated on the lower leaf surface (Sampson 1961) under a light microscope (*Leica DMLB*, Houston, USA) fitted with a *Leica 500 MC* computer. Experiments were applied using a completely randomized design and data were statistically analyzed using *Minitab Version 11 for Windows*. Means were compared using LSD test (Clewer and Scarisbrick 2001).

It seems that relative humidity inside NVV was higher than that inside VV as water droplets frequently appeared on their walls. Shoot primordia developed directly or indirectly. Callus developed in NVV was white and friable although later some of them turned brown and died, but it was compact and white or green-white in VV. Adventitious shoots did not elongate on SIM but they elongated and rooted when subcultured on SEM. On that medium, NVV-maintained shoots had callus-like structures on their leaves and stems. Nevertheless, this structure did not develop further and disappeared once shoots were subcultured onto rooting medium. The number of developing shoots was significantly affected by vessel type and PGRs. Ventilation significantly increased the percentage of explant which developed shoots; however, PGR combinations affected this percentage. Shoot primordia which developed in NVV containing medium with 11 µM IAA + 12, 24 and 36 µM BA and 22 µM IAA + 12 µM BA failed to show further development during the shoot elongation stage. On the other hand, 1.7 - 6.2 shoot per explant regenerated in VV using the previous combinations of PGRs. These results regarding callus texture are in contradiction to those of Zobayed *et al*. (1999b) which showed that ventilation induced friable callus. However, they stated that callus in sealed vessels had lower volumes with some necrotic spots. Nguyen *et al*. (2007) observed good growth of *Sorghum bicolor* callus under ventilation. The effect of ventilation on callus growth and regeneration could be attributed to the accumulation of ethylene in the culture head-space (Adkins *et al*. 1990, Zobayed *et al*. 1999a). The effect of growth regulators on callus and shoot regeneration of peppers was reviewed by Kothari *et al*. (2010).

A high number of shoots was achieved following subculture onto medium containing a low concentration of 2iP. Moreover, there were significant differences in the number of shoots per explant in response to vessel type and PGRs. Under non-ventilated conditions explants cultured on SIM containing 24 or 36 µM BA + 22 µM IAA developed the highest number of shoots (6.5 - 7.2 shoots per explant) compared with other PGRs combinations (Table 1). However, using the same PGR combinations, explants maintained in VV had significantly higher shoot number than those maintained in NVV. The highest rate of shoot organogenesis (9.8 and 11.5 shoots per explant) was achieved in VV with SIM enriched with 22 µM IAA + 36 µM BA and 22 µM IAA + 24 µM BA, respectively (Table 1). These shoot numbers are considerably higher than those previously obtained by Ochoa-Alejo and Ireta-Moreno (1990) with

Table 1. Effect of vessel type and PGRs on shoot induction, shoot and root length, fresh and dry mass, stomata number and contents of chlorophylls and soluble sugars in *C. annuum* cv. Anaheim after 4 weeks culturing on rooting medium. Explants were cultured on SIM with IAA and BA for 3 months and then subcultured onto SEM with IAA + 2iP for 2 months. Finally shoots were transferred onto MS medium with 9 μM of IBA for rooting.

Vessel	IAA+BA/ IAA+2iP [μM]	Shoot number [explants^{-1}]	Shoot length [mm]	Root length [mm]	FM [g plant^{-1}]	DM [mg plant^{-1}]	Stomata number [mm^{-2}]	Chl *a* [μg g^{-1}(FM)]	Chl *b* [μg g^{-1}(FM)]	TSS [μg g^{-1}(DM)]
VV	0+24/ 0+9	5.2	5.2	5.6	2.76	336	9.5	56	36	614
	0+36/ 0+9	5.7	6.8	5.8	2.52	406	12.5	54	41	641
	22+24/22+9	9.8	6.4	7.0	1.64	409	10.2	40	29	605
	22+36/22+9	11.5	6.4	6.5	1.60	449	13.5	48	31	652
NVV	0+24/ 0+9	2.1	6.9	7.0	4.02	261	30.5	44	33	618
	0+36/ 0+9	2.8	7.5	6.3	3.49	288	25.8	48	35	600
	22+24/22+9	6.5	8.0	9.7	2.04	253	29.1	32	21	627
	22+36/22+9	7.2	8.0	7.9	2.84	139	26.6	39	24	640
$LSD_{0.05}$		1.1	ns	1.5	0.35	43	2.1	5	4	ns

hypocotyl and MS medium with BA, 2iP and IAA, indicating that cotyledons have higher regeneration capacity than hypocotyls even when cultured in NVV. Low concentrations of BA and IAA enhanced shoot elongation of *C. anuum* (see Kothari *et al*. 2010). Subculturing on medium with low concentrations of PGRs enhanced rooting, and almost all shoots transferred onto rooting medium were elongated and rooted. Cytokinins commonly stimulate shoot proliferation but inhibit its elongation. Our results are consistent with those of Sanatombi and Sharma (2008) as shoot were elongated and rooted on medium containing 9 μM IBA. Some plantlets maintained in VV formed 3 - 5 flowers which lasted for 10 - 15 d, whereas in NVV flowering buds were formed but they become brown and died at an early stage. There were no significant differences in plantlet height among regenerated plantlets; however, those grown in NVV had significantly longer root and higher FM than VV-grown ones using the same combinations of PGRs. The highest plantlet FM (4.02 g per plantlet) was in NVV containing SIM with 24 μM BA. However, the highest DM (449 mg per plantlet) was recorded in VV using medium with 22 μM IAA + 36 μM BA during shoot induction and 22 μM IAA + 9 μM 2iP for shoot elongation stage. This value is approximately three-fold that of plantlets grown on the same medium using NVV (Table 1). The highest DM and lowest FM of VV-developing plantlets indicates the lower moisture content of the tissue in plantlets grown in VV. Increasing FM in NVV might be due to the higher water retention related to the reduced evapotranspiration which is affected by limited air exchange (Haisel *et al*. 1999). Higher DM in VV has been found on sweet potato (Yulan and Toyoki 2006) as ventilation promoted diffusion of CO_2 and water vapor. The increase of CO_2 and water vapor diffusion promotes photosynthesis, transpiration and *in vitro* plantlets growth (Solárová and Pospísilová 1997). The *in vitro* growth of plantlets is often greater under photoautotrophic conditions than under heterotrophic conditions, provided that the *in vitro* environment is properly controlled for promoting photosynthesis (Hazarika 2006).

Vessel type and PGRs had significant effects on the photosynthetic pigment content of leaves. Plantlets grown in VV had higher contents of chlorophyll (Chl) *a,* Chl *b* and total Chl than NVV ones using the same growth media (Table 1). The highest content of total Chl (124 μg g^{-1}) was achieved in VV and SIM supplemented with 9 μM BA whereas plantlets developed in NVV on medium with 22 μM IAA + 36 μM BA during shoot induction and 22 μM IAA + 9 μM 2iP during shoot elongation had the lowest total Chl content (62 μg g^{-1}). In all cases, medium enriched with IAA reduced the leaf Chl contents. Before acclimatization TSS content varied between 600 and 627 μg g^{-1} with no significant differences among plantlets (Table 1). Hazarika (2006) considered that ventilation and low sucrose concentration would make the photosynthetic apparatus more developed and thus Chl content and net photosynthetic rate were higher (Haisel *et al*. 1999).

Leaves of NVV-micropropagated plantlets had significantly more stomata than those in VV using the same PGRs concentrations. The highest and lowest densities of stomata (30.5 and 9.5 mm^{-2}) were found in plantlets grown on medium supplemented with 24 μM BA in NVV and VV, respectively (Table 1). Moreover, closed spherical stomata were observed under ventilation but NVV-developing plantlets had elliptical open stomata. The high relative humidity and ethylene accumulation in closed vessels might cause the development of stomata with abnormal density and shape which are not able to close (Khan *et al*. 2003, Mohamed and Alsadon 2010)

Ex vitro survival rate of VV-micropropagated plantlets (75 - 86 %) was significantly higher than that of NVV ones (44 - 61 %) and there was no significant influence of PGRs on the survival rate. Stomatal density

Table 2. Effect of vessel type and PGRs during micropropagation on survival rate, fresh and dry mass, density of stomata, and contents of Chl *a*, Chl *b* and TSS in *C. annuum* cv. Anaheim plantlets after 4 weeks of acclimatization. Explants were cultured on SIM for 3 months and then subcultured onto SEM for 2 months. Finally shoots were transferred onto MS medium with 9 μM IBA for rooting.

Vessel	IAA+BA/ IAA+2iP [μM]	Survival [%]	FM [g plant^{-1}]	DM [mg plant^{-1}]	Stomata number [mm^{-2}]	Chl *a* [μg g^{-1}(FM)]	Chl *b* [μg g^{-1}(FM)]	TSS [μg g^{-1}(DM)]
VV	0+24/ 0+9	86	9.56	3.65	11.6	59	34	799
	0+36/ 0+9	75	10.05	3.91	14.7	45	36	790
	22+24/22+9	81	8.89	3.25	12.9	48	25	845
	22+36/22+9	80	8.91	3.71	15.0	44	32	832
NVV	0+24/ 0+9	55	7.02	2.21	25.9	52	40	708
	0+36/ 0+9	61	7.49	2.54	20.5	40	40	755
	22+24/22+9	49	7.34	2.95	22.8	40	32	712
	22+36/22+9	58	6.54	2.44	19.2	39	36	700
$LSD_{0.05}$		16	1.51	0.48	2.3	5	8	52

and characteristics of NVV-developing plantlets could influence the survival rate during acclimatization by enhancing transpiration whereas ventilation decreased stomatal density and resulted in more functional stomata (Hazarika 2006). Plants developed in VV flowered after acclimatization unlike NVV-propagated ones. Although NVV-micropropagated plantlets had higher FM than VV-ones before acclimatization their *ex vitro* FM was significantly lower than that of plantlets micropropagated in VV conditions. The FM of VV-grown plantlets varied between 8.91 and 10.05 g per plant with no significant difference among treatments. Plantlet DM had a trend similar to that before acclimatization but with significant differences among VV and NVV treatments which reflect the significant interaction between PGRs and vessels type. These findings are in agreement with Lian *et al*. (2002) who found that photoautotrophic conditions improved *ex vitro* percent survival and Chl and sugar contents by enhancing net photosynthetic rate. Solárová and Pospíšilová (1997) suggested that *in vitro* grown plantlets need more CO_2 which could be facilitated using gas permeable closures.

After acclimatization, VV-developing plantlets still had fewer stomata (11.6 - 15.00 mm^{-2}) than NVV ones (Table 2). The new developing leaves of all plantlets which acclimatized to the *ex vitro* conditions had spherical stomata similar to those of greenhouse grown plants. Leaf stomatal density of NVV-maintained plantlets decreased from 25.8 - 30.5 to 19.2 - 25.9 mm^{-2} after acclimatization. The reduction in stomatal density and the changes in stomatal shape after acclimatization have been attributed to correction of abnormalities induced by *in vitro* conditions (Pospíšilová *et al*. 1999).

There was no significant effect of ventilation and PGRs on photosynthetic pigments after acclimatization; however, there was significant interaction between the two factors. A similar trend of Chl *a* and Chl *b* contents were observed as before acclimatization (Tables 1 and 2). Plantlets micropropagated in VV had significantly higher TSS content (790 - 832 μg g^{-1}) than those developed in NVV (700 - 755 μg g^{-1}) except those grown on SIM with 36 μM BA. The poor growth and low survival percentage of plantlets developed in NVV can be also attributed to the inhibitory effect of ethylene accumulation (Zobayed *et al*. 1999a). Moreover, improving growth parameters and higher TSS content of VV-developing plantlets after acclimatization could be a function of a balanced CO_2 supply enabling the plants to benefit from higher net photosynthetic rate and production of assimilates (Zobayed *et al*. 1999b).

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