

The Production of Cauliflower Microshoots Using Curd Meristematic Tissues and Hypocotyl-Derived Callus

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Abstract

The capacity for microshoot production from cauliflower was investigated applying two different protocols. In the first, cauliflower curd meristematic tissue was used as a source of explants. The meristematic layer was shaved off and the clusters produced were homogenised using a commercial blender. In terms of its effect on the number and viability of microshoots, the use of 30 s blending duration treatment was found to be optimal between several treatments tested in the range 15 to 120 s. Explants were cultivated in agitated S23 (MS (Murashige and Skoog, 1962) + 3% sucrose) liquid media supplemented with different combinations of plant growth regulators. The use of 2 mg/L kinetin and 1 mg/L IBA gave the optimal results in terms of the number and viability of microshoots. The second protocol was designed to investigate the regeneration potential of hypocotyl explants of cauliflower via callus culture. The callus tissue was initiated from hypocotyl explants in callus induction medium (CIM), which consisted of S23 supplemented with 2,4-D at 1 mg/L and kinetin at 1.5 mg/L. The highest number of shoots was obtained after 28 days from sub-cultured hypocotyl derived callus on S23 basal media containing 0.5 mg/L of kinetin. This study demonstrated the ability of producing microshoots using various parts of cauliflower through both callus and without callus formation, which can be useful in the later applications of cauliflower tissue culture such as the production of artificial seeds.

INTRODUCTION

In vitro shoot propagation from *Brassica* seedling explants is interesting because of a plentiful supply of responsive explants from many plant structures including cotyledons, seedling roots, hypocotyls, stem sections, petals and leaf tissue (Cao, 2003) and regeneration of plants has been achieved from various tissues (Hachey et al., 1991). Shoot formation with high proliferation frequencies has been achieved from hypocotyl explants of commercial cauliflower genotypes (Bhalla and Smith, 1998). Different types of plant materials such as, seedling explants (Arora et al., 1997), anther culture (Fuller et al., 1990), protoplast culture (Yang et al., 1994), roots (Horeau et al., 1988; Narasimhulu and Chopra, 1988) and peduncles (Christey and Earle, 1991) have been reportedly used for cauliflower micropropagation. In vitro propagation favours the culture of pre-existing meristems because of their superior genetic stability and the cauliflower curd has a great advantage, since it contains millions of meristems forming an ideal basic material for tissue culture applications (Kieffer et al., 2001).

This study aimed to investigate the ability of using both meristematic and hypocotyl tissues for cauliflower microshoot production.

MATERIAL AND METHODS

Three F₁ hybrid cultivars of cauliflower, 'Clemen', 'Broden' and 'White Cloud' previously shown in our laboratory to be equally responsive in tissue culture (unpublished) were used. Plants of 'Clemen' and 'Broden' were obtained from the field in

Cornwall courtesy of Simmonds & Sons Ltd. and replanted in raised beds at University of Plymouth. The plants were grown according to good commercial practice and raised to maturity when the curds were harvested and stored at 2-4°C until required. Seeds of 'White Cloud' were used to produce seedlings, which were then used for the production of hypocotyl explants.

Cauliflower Microshoot Production

Large pieces (1-5 cm) of cauliflower curds were sterilized by immersion in 10% by volume un-thickened domestic bleach (0.06% sodium hypochlorite) for 15 min followed by a double wash with sterile distilled water. Explants were produced mechanically by eliminating the mass of non-responsive tissue (stem branches) and shaving off the upper meristematic layer. The meristematic clusters were then homogenized using a commercial blender (Waring model 800) at approximately 1700 rev min⁻¹ in S23 liquid medium. Eight blending durations in the range 15-120 s were assessed in terms of their effects on the amount and viability of explants produced. Five replications, each consists of 100 g of meristematic clusters, were used for every blending treatment. The "soup" produced was sieved through precision sieves (212, 300 and 600 µm) (Endecotts Ltd., London, UK). The micro-explants were collected off the sieves, weighed and converted to aliquots of explants using small precision volumetric measures (240±2 µl) and the average weight and volume of explants produced were determined.

Six containers (150 ml plastic pots), each containing 30 ml liquid culture medium supplemented with 2 mg/L Kinetin, 1 mg/L IBA and 1 ml/L of PPM (plant preservative mixture) were cultured with a constant volume of explants (74 µl) and used with each blending duration in order to assess the effect of blending treatments on the number and viability of microshoots developed at the various size classes. The pots were constantly shaken (150 rev min⁻¹) during culture at 20°C and exposed to 16 h photoperiod (80 µmol m⁻² s⁻¹) in the plant physiology laboratory at the University of Plymouth.

Nine combinations of plant growth regulators consisting of the use of 1 and 2 mg/L kinetin incorporated with three types of auxins including 1 and 2 mg/L of IBA (indole butyric acid), 1 and 2 mg/L of NAA (naphthalene acetic acid) or 1 and 2 mg/L of IAA (indole-3-acetic acid) were used with the culture medium to investigate their effects on the number and viability of microshoots. Five pots, each containing 30 ml of culture medium were cultivated with a constant volume (74 µl) of 300-600 µm explants and used with each treatment. The number and viability of microshoots were recorded after 15 days of culture.

Three methods of agitation were tested in terms of their effects on explant growth and viability using 300-600 µm produced explants (30 s blending treatments) in liquid media consisting of: S23 supplemented with 2 mg/L kinetin, 1 mg/L IBA and 1 ml/L PPM. 1) Four containers, each with 74 µl of explants, were set in an orbital shaker at 150 rev min⁻¹ (Fig. 1). The number of microshoots and their viability assessed as average fresh weight were evaluated after 22 days culture. 2) Four culture containers, each with 74 µl of explants, were supplied with a current of air bubbles produced by an air pump (Hozelock cyprio, model 1380-0000). The air was purified using a millipore air filter (Millex HA, 0.22 µm) (Fig. 1). The number of microshoots and their viability as average fresh weight were evaluated after 29 days of culture. 3) Cultivated explants were agitated both by a magnetic stirrer and simultaneously by a current of air bubbles. 296 µl of explants were cultured in 120 ml of liquid media contained in 1-L size flask, which was the same explants:medium density as the other treatments.

Cauliflower Seedling Explants Production

Mature seeds were surface sterilized in commercial sodium hypochlorite solution (NaOCl) 50% (v/v) for 10 min with 2 drops of Tween80, followed by three times rinses with sterile distilled water and then immersed in 90% ethanol for 1 min followed by three rinses with sterilized distilled water. The surface sterilized seeds were germinated on PGR (plant growth regulator) free S23 (basal medium 4.4 g/L, 30 g/L sucrose, 7 g/L agar). The

seeds were incubated in 16 hours light provided by fluorescent lights) ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) / 8 hours at 22.5°C . Hypocotyl segments 5 mm in length were excised from 7-10 day old seedlings in a laminar flow cabinet as described by Fuller and Fuller (1995).

Callus tissue was initiated from hypocotyl explants on callus induction medium (CIM) which consisted of S23 supplemented with 2,4-D at 1 mg/L and kinetin at 1.5 mg/L. Callus was excised carefully to ensure no original explant material was included in the sub-culture. This was then cut into small pieces (2 mm diameter) and sub-cultured on shoot induction media (SIM) as follows A) free PGR; B) 0.5 mg/L 2,4-D and 0.5 mg/L kinetin; C) 0.5 mg/L 2,4-D and 0.5 mg/L kinetin; D) 1.5 mg/L kinetin. 3 pieces of callus were placed in each 9 cm petri dish with 5 replicates for each treatment. Shoot growth was assessed over 28 days and the number of shoots recorded.

Results are presented as means + standard error (S.E.). All data were subjected to analysis of variance (ANOVA) using Minitab software (version 15) and comparisons of means were made with least significant difference test (LSD) at 5% level of probability.

RESULTS

The maximum number of growing explants at size class 212-300 μm were produced using 30 s blending duration treatment with a highly significant difference ($P < 0.001$) compared with other blending treatments. For size class 300-600 μm , 15 s blending duration was found to be optimal giving the highest number of developing explants ($P < 0.001$) (Fig. 2).

In terms of microshoot viability, although the maximum microshoot average weight ($P < 0.001$) was produced using 60 s blending duration at size class 300-600 μm (Fig. 2), the number of growing explants was very low. The maximum average weight of produced microshoots (fast growing) was obtained using 15 s blending duration treatment ($P < 0.001$) at size class 212-300. However, no significant differences were obtained between the use of 15 and 30 s treatments in terms of viability (fresh weight) of microshoots produced. While no significant difference was observed between the number of microshoots produced using 15 and 30 s blending treatments at size class 300-600 μm , the number of developing microshoots was significantly higher using 30 s treatment compared with the use of 15 s at size class 212-300 μm (Fig. 2).

Highly significant differences were observed between the use of 15 and 30 s blending treatment on the total amount of explants produced in terms of both volume and weight at size class 212-300 μm (Fig. 3). The use of 30 s blending treatment was recommended as being optimal.

In terms of the effect of PGRs used in the culture medium, 2 mg/L of kinetin and 1 mg/L of IBA were found to be optimal in terms of both number and viability of microshoots produced.

The use of the orbital shaker at 150 rev/min gave better results in terms of the number of developing microshoots and their viability measured as fresh weight. The number of developing microshoots was found to be 360 and 55 per pot using orbital shaker and current of air bubbles respectively. Moreover, the level of microshoot hyperhydricity was high using the current of air bubbles as an agitation method (Fig. 4).

It was observed that the use of a current of air bubbles delayed the growth of the explants. Thus, the number and the viability of microshoots were evaluated after 29 days of culture rather than the normal 22 days applied with shaker agitation. Moreover, the amount of culture media used with this agitation method was much higher than that used when the orbital shaker was employed. It was also necessary to add some liquid medium every few days to keep the culture media at the required level since the air bubbles accelerated evaporation of the water from the medium. None of the cultivated explants cultured using a current of air bubbles and magnetic stirrer developed into shoots. This suggested that the magnetic stirrer might damage explants or that the topping up of the medium caused a toxic accumulation of mineral salts. Therefore, the use of an orbital shaker as an agitation method is recommended for mass microshoot production.

Shoots were regenerated from hypocotyl-derived callus and the maximum

($P < 0.001$). Number of shoots was on medium containing kinetin at 0.5 mg/L (Fig. 5). A significant response also occurred on media with 0.5 mg/L 2,4-D + 0.5 mg/L kinetin and 1.5 mg/L kinetin compared with PGR free medium.

DISCUSSION

The use of a blender for the mass production of cauliflower explants was found to be a highly effective method for providing a huge amount of uniform explants. The use of blenders has also been described for mass production of initial explants of fern (Janssens and Sepelie, 1989) and for separating meristemoid aggregates of several species (Ziv et al., 1998). The use of a blender for cauliflower curd explant production was also previously reported by Kieffer et al. (2001).

Liquid culture has a tendency to produce in vitro cultures with enhanced hyperhydricity but in the current study this was only observed when air bubbles were used as a means of agitation. The use of a current of air bubbles is supposed to reduce the level of hyperhydricity because it decreases the level of accumulated ethylene, which is considered as one of the main reasons behind this physiological phenomenon (Akhter Zobayed et al., 1999). In contrast our results indicated that the level of hyperhydricity was higher using a current of air bubbles released in the liquid media when compared to the use of orbital shaker for agitation. Thus, hyperhydricity in cauliflower in vitro culture must be caused by another factor such as the presence of a high level of cytokinins (kinetin) for long periods. Furthermore when the current of air bubbles was used, the growth of explants was delayed significantly and the amount of fresh liquid media used was much higher because of the loss of liquid caused by enhanced evaporation. Therefore, there could have been a concentration effect of plant growth regulators over the period of the experiment. High levels of cytokinins are known for their effect of raising the level of hyperhydricity (Ivanova and van Staden, 2008). Although several studies have investigated the effect of the cytokinins on the hyperhydricity phenomenon (Thomas, 1999), the function of plant growth regulators in hyperhydricity has yet to be fully determined and evidence exists for their effects on regular or hyperhydric morphogenesis is still controversial (Picoli et al., 2001). Despite this, the use of an agitated liquid culture medium by means of a shaker appeared to allow an optimal shoot regeneration frequency and growth rate in accordance with previous reports (Grout and Crisp, 1977).

The results showed that the supplementation with plant growth regulators was an essential requirement for the developmental induction of the cauliflower explants and it is known that cytokinins (i.e., kinetin) have a crucial position in the organization of sink activity and nutrient partitioning (Kuiper, 1988; Kuiper et al., 1989). The various differences obtained between the auxins types tested gave a clear idea that auxin has a role in the induction of cauliflower explants. This is in contrast to Vandemoortele et al (2001) who commented that it is difficult to associate endogenous auxins with a function in the induction of cauliflower curd explants.

A high efficiency of shoot regeneration from hypocotyl-derived callus was obtained when explants were placed on medium containing cytokinin compared to the medium free of PGRs. Our findings are in agreement with (Burbulis et al., 2009) who reported that media containing cytokinins alone can promote shoots on explants of rapeseed at higher frequency compared to the media devoid of growth regulators. Ahmed and Spoor (1999) also mentioned that the induction of shoots can be achieved by high cytokinin with low or no auxin concentrations. The current results showed that the use of kinetin is probably the most important PGR in terms of shoot formation in cauliflower explants.

CONCLUSION

Cauliflower microshoots were optimised using both curd meristematic layer (without callus formation) and hypocotyls-derived callus. The use of the meristematic layer protocol was more effective in terms of the number of microshoots and the

reduction of the time required to the end point of shoot production. Kinetin was an essential plant growth regulator for shoot induction. Further studies are required to investigate the effect of microshoot resources on the quality of microshoots and their growth capacity in subsequent stages towards in-vivo plant production.

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Literature Cited

- Ahmad, S. and Spoor, W. 1999. Effect of NAA and BAP on callus culture and plant regeneration in curly Kale (*Brassica oleraces* L.). Pak. J. Biol. Sci. 2:109-112.
- Akhter Zobayed, S.M., Armstrong, J. and Armstrong, W. 1999. Cauliflower shoot-culture: effects of different types of ventilation on growth and physiology. Plant Sci. 14:209-217.
- Arora, N., Yadav, N.R., Yadav, R.C. and Chowdhury, J.B. 1997. Role of IAA and BAP on plant regeneration in cultured cotyledons of cauliflower. Cruciferae Newsl. 19:41-42.
- Bhalla, P.L. and Smith, N.A. 1998. Comparison of shoot regeneration potential from seedling explants of Australian cauliflower (*Brassica oleracea* var. *botrytis*) varieties. Aust. J. Agr. Res. 49:1261-1266.
- Burbulis, N., Blinstrubiene, A., Kupriene, R., Jonytiene, V., Rugienius, R. and Staniene, G. 2009. In vitro regeneration of *Brassica napus* L. shoots from hypocotyls and stem segments. Zemdirbyste-Agriculture 96:176-185.
- Cao, J. and Earle, E.D. 2003. Transgene expression in broccoli (*Brassica oleracea* var. *italica*) clones propagated in vitro via leaf explants. Plant Cell Rep. 21:789.
- Christey, M.C. and Earle, E.D. 1991. Regeneration of *Brassica oleracea* from peduncle explants. HortScience 26:1069-1072.
- Fuller, M.P., Turton, S. and Grout, B.W.W. 1990. Anther culture of winter-heading cauliflower. Acta Hort. 280:329-332.
- Grout, B.W.W. and Crisp, P. 1977. Practical aspects of the propagation of cauliflower by meristem culture. Acta Hort. 78:289-296.
- Hachey, J.E., Sharma, K.K. and Moloney, M.M. 1991. Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured in vitro. Plant Cell Rep. 9:549-554.
- Horeau, N., Arona, R. and Bhojwani, S.S. 1988. A comparative study of in vitro shoot regeneration from cotyledon and root explants of four varieties of *Brassica oleracea* L. Curr Sci. 57:1349-1351.
- Ivanova, M. and van Staden, J. 2008. Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of in vitro regenerated shoots of *Aloe polyphylla*. Plant Cell Tiss Org. 92:227-231.
- Janssens, J. and Sepelie, M. 1989. Rapid multiplication of ferns by homogenization. Scientia Horti. 38:161-164.
- Kieffer, M., Simkins, N., Fuller, M.P. and Jellings, A.J. 2001. A cost effective protocol for in vitro mass propagation of cauliflower. Plant Sci. 160:1015-1024.
- Kuiper, D. 1988. Growth responses of *Plantago major* L. ssp. *pleiosperma* (Pilger) to changes in mineral supply: evidence for regulation by cytokinins. Plant Physiol. 87:555-557.
- Kuiper, D., Kuiper, P.J.C., Lambers, H., Schuit, J. and Staal, M. 1989. Cytokinin concentration in relation to mineral nutrition and benzyladenine treatment in *Plantago major* ssp. *pleiosperma*. Physiologia Plantarum 75:511-517.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
- Narasimhulu, S.B. and Chopra, V.L. 1988. Species-specific shoot regeneration response

- of cotyledonary explants of brassicas. *Plant Cell Rep.* 7:104-106.
- Picoli, E.A.T., Otoni, W.C., Figueira, M.L., Carolino, S.M.B., Almeida, R.S., Silva, E.A.M., Carvalho, C.R. and Fontes, E.P.B. 2001. Hyperhydricity in in vitro eggplant regenerated plants: structural characteristics and involvement of BiP (Binding Protein). *Plant Sci.* 160:857-868.
- Thomas, T.H. 1999. *The Physiology of Vegetable Crops.* Edited by H.C. Wien. 1997. *J. Plant Growth Regul.* 27:137-138.
- Vandemoortele, J.L., Kevers, C., Billard, J.-P. and Gaspar, T. 2001. Osmotic pretreatment promotes axillary shooting from cauliflower curd pieces by acting through internal cytokinin level modifications. *J. Plant Physiol.* 158:221-225.
- Yang, Z.-N., Xu, Z.-H. and Wei, Z.-M. 1994. Cauliflower inflorescence protoplast culture and plant regeneration. *Plant Cell Tiss Org.* 36:191-195.
- Ziv, M., Ronen, G. and Raviv, M. 1998. Proliferation of meristematic clusters in disposable presterilized plastic bioreactors for the large-scale micropropagation of plants. *In Vitro Cell Dev. Biol. Plant.* 34:152-158.

Figures

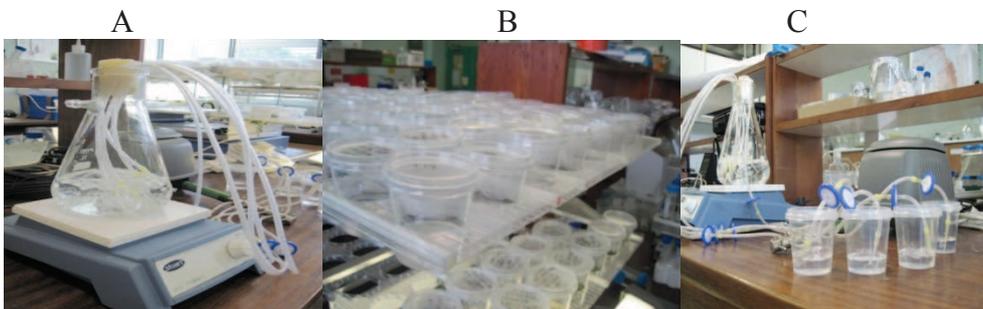


Fig. 1. Explants production and agitation methods: A) orbital stirrer; B) air bubbles; C) air bubbles and magnetic stirrer.

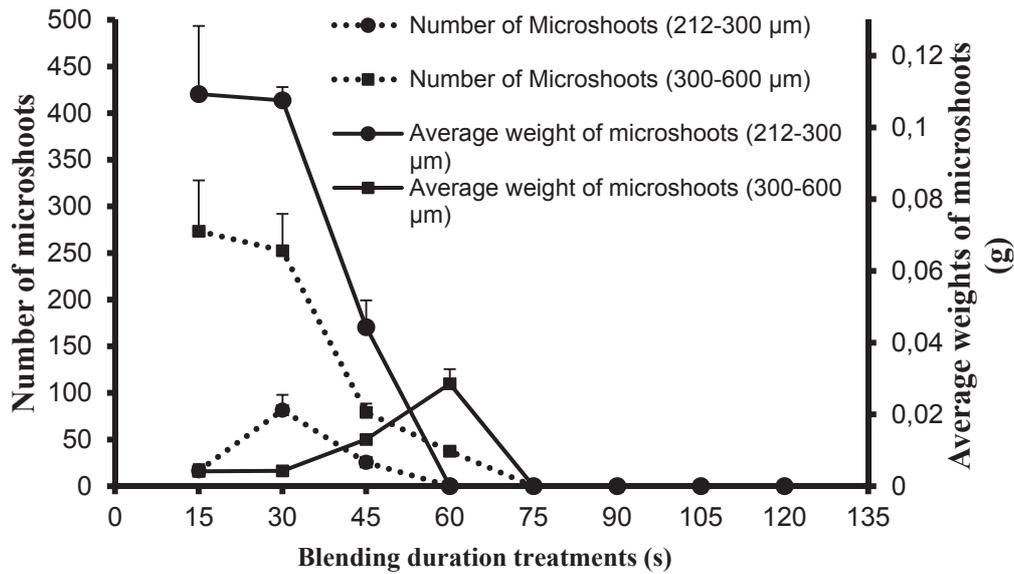


Fig. 2. The effect of the blending duration treatments on the number of growing microshoots (LSD=19.58 for 212-300 µm size class, LSD=70.52 for 300-600 µm size class) and their average weights at two size classes, 212-300 and 300-600 µm (LSD=0.022 for 212-300 µm size class, LSD=0.0045 for 300-600 µm size class).

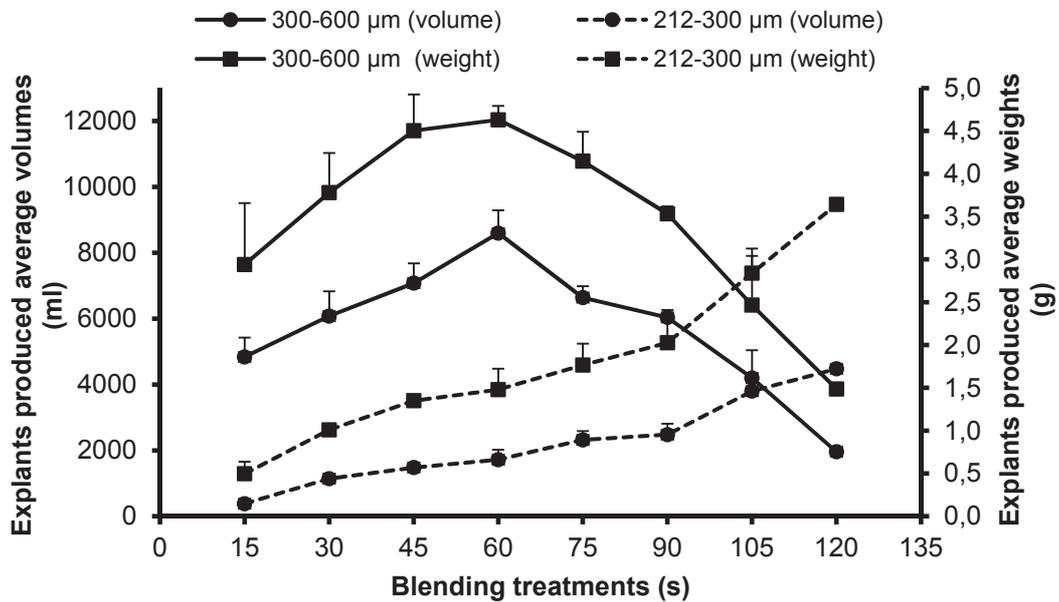


Fig. 3. The effect of blending duration on explant produced weight (g) (LSD=1.171 for 212-300 µm, LSD=0.167 for 300-600 µm size class) and volume volumes (LSD=756.74 for 212-300 µm, LSD=1626.43 for 300-600 µm size class).



Fig. 4. Hyperhydricity as a result of cultivation using air bubbles as an agitation method.

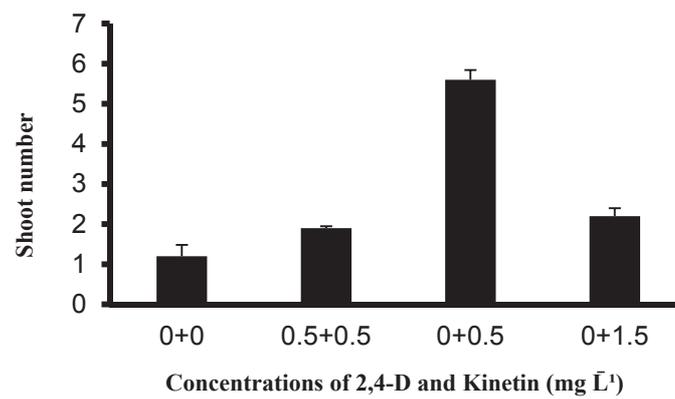


Fig. 5. The effect of exogenous hormones (2, 4-D mg L⁻¹ and Kinetin mg L⁻¹) on shoot formation after 28 days of culture (LSD=0.48).