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The effect of using PPM (plant preservative mixture) on the development of cauliflower microshoots and the quality of artificial seed produced

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ABSTRACT

The effects of using PPM (plant preservative mixture) (Apollo Scientific Limited, UK) on the growth of cauliflower microshoots were determined. A negative correlation was found between the concentration of PPM in the liquid medium and the number of microshoots developed: the greater the concentration, the lower microshoot number. The stage of the culture process most suitable for the introduction of PPM was also investigated. While the use of PPM with blending medium (S23: 4.4 g L^{-1} MS + 30 g L^{-1} sucrose) did not control the later contamination in the culture medium, the use of 0.5 mL L^{-1} of PPM with culture medium (S23 supplemented with 2 mg L^{-1} (9.29 μM) of kinetin + 1 mg L^{-1} (4.9 μM) of IBA (indole butyric acid)) was found to be effective in controlling contamination and keeping the growth capacity of microshoots. Cauliflower microshoots were encapsulated in sodium alginate as artificial seeds. Artificial seeds conversion rate and viability assessed as fresh weights of plantlets produced were evaluated in different culture substrates (compost, perlite, sand and vermiculite). The effects of PPM concentrations used with S23 irrigation solutions were also evaluated. This study showed the effectiveness of using PPM in controlling the contamination and the necessity for determination the correct concentration and the correct stage for the use of this material in order to obtain optimum results.

1. Introduction

Kieffer et al. (2001) designed an effective protocol for cauliflower micropropagation involving the use of a commercial blender and sieves for the production of explants and this has recently been revised by Rihan et al. (2011). From this process it has been shown that cauliflower explants have a high capacity to develop to microshoots suitable to be encapsulated as artificial seeds. The technique of artificial seed has been widely studied and works with various plant species including fruits, cereals, medicinal plants, vegetables, ornamentals, forest trees and orchids (Antonietta et al., 2007; Germanà et al., 2011; Malabadi et al., 2005; Pintos et al., 2008; Rai et al., 2008; Singh et al., 2009; Sundararaj et al., 2010). The use of microshoots has been widely reported for the production of artificial seeds in different plant species such as *Cineraria maritime* and *Musa* sp. (Sandoval-Yugar et al., 2009), *Gerbera jamesonii* (Taha et al., 2009) and *Picrorhiza kurrooa* (Mishra et al., 2010).

The propagation of cauliflower clones reproduced by tissue culture and distributed as artificial seeds might be useful alternative to costly F1 hybrids varieties and useful in the terms of the conservation of elite clonal germplasm in cauliflower breeding programmes. There is however, a high risk of contamination during the protocol despite strict sterilized conditions which are normally imposed. Contamination by bacteria, fungi and yeasts is a constant challenge that frequently threatens plant tissue cultures during the culture phase (Leifert and Cassells, 2001). Several studies have investigated the elimination of bacterial and fungal contamination in culture systems using various materials including the use of fungicidal (Haldeman et al., 1987) and antibiotic treatments (Kneifel and Leonhardt, 1992; Leifert et al., 1992). However, although the use of these materials can be effective for controlling or restraining contamination (Reed and Tanprasert, 1995), two essential limitations have been encountered in their use firstly, the reduction of a microorganisms sensitivity to these substances, i.e. building up of resistance (Seckinger, 1995) and secondly, their inactivation caused by heat during autoclaving (Barrett and Cassells, 1994).

Plant preservative mixture (PPM) (Apollo Scientific Limited, UK) is a combination of two broad-spectrum industrial isothiazolone biocides, chloromethylisothiazolone and methylisothiazolone.

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PPM is heat stable and is thus able to be autoclaved with the culture medium (Lunghusen, 1998). Resistant mutants toward PPM are unlikely to be formed since PPM targets specific multiple enzyme sites in the Krebs cycle and the electron transport chain of micro-organisms (Chapman and Diehl, 1995). As with any anti-microbial compound there is a risk that it is phyto-toxic and checks need to be made to ensure that they do not inhibit or alter plant growth during in vitro culture or in subsequent in vivo establishment.

Niedz (1998) has tested PPM with many types of citrus tissue culture and demonstrated that PPM could be used with the culture media to control bacterial and fungal contamination without impairing the plant material. Also, Fuller and Pizzey (2001) indicated the important role of PPM to control the contamination in brassicas culture media. The effects of PPM on several species have been studied for example, non-embryogenic callus of sweet orange (*Citrus sinensis* (L.) Osbeck 'Valencia'), shoot regeneration of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and rough lemon (*Citrus jambhiri* Lush.) (Niedz, 1998), adventitious melon, petunia and tobacco (Compton and Koch, 2001), and pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.) (Guri and Patel, 1998). All of these studies demonstrated the positive effect of PPM to control the contamination and highlighted the importance of using PPM at a proper concentration which was indicated to be different depending on the plant species since the use of PPM at relatively high concentration could have negative effects on the development of plant tissue.

This current study aimed to determine the best concentration of PPM for limiting the contamination of cauliflower microshoot production without affecting explant growth ability, and also the impact of the presence of PPM at particular growth stages, i.e. whether the presence of the PPM in the blending medium (S23) (the medium used in the blending stage for the homogenizing of curd clusters) is enough or whether it is necessary for the PPM to be in the culture medium in order to control contamination. However, only visible contamination screening was completed and the culture which visually appeared "sterile" could be contaminated because the PPM is considered to be a bacteriostatic agent. Microshoots were then used for the production of artificial seeds and this study also aimed to investigate the effect of using PPM with liquid medium applied as fertigation during artificial seed conversion in various commercial growing substrates (compost, perlite, sand and vermiculite).

2. Materials and methods

Two varieties of cauliflower, Dionis and Mascaret, were obtained from the field courtesy of Simmonds & Sons Ltd. (West Cornwall, Devon, UK) and replanted in raised beds at the University of Plymouth. The plants were grown according to standard commercial practice (Whitwell et al., 1982) and raised to maturity when the curds were harvested and stored at 2–4 °C until required. The use of 2 varieties gave a continuous supply of cauliflower heads over the experimental period.

2.1. Cauliflower microshoot production

Large pieces (1–5 cm) of curd were surface sterilized by immersion in 10% by volume domestic bleach (0.06% sodium hypochloride), thin basic domestic bleach, Sainsbury, UK) for 15 min followed by a double wash with sterile distilled water. Explants were produced in a laminar flow hood by mechanically eliminating the mass of non-responsive tissue (stem branches) and shaving off the upper meristematic layer using a sterilized scalpel. The meristimatic clusters were then homogenized using a commercial blender (Waring model 800, UK) at approximately 1700 rev min^{-1} in maintenance S23 liquid medium (blending medium) (MS 4.4 g L^{-1} (Murashige and Skoog, 1962) (Sigma, M5519-50L) + sucrose 30 g L^{-1}) for 30 s. The Blending medium containing the micro-explants was then sieved through precision sieves (212, 300 and 600 μm) (Endecotts Ltd., London, UK). The micro-explants were collected from the sieves, weighed and converted to aliquots of explants, for subsequent culture, using small precision volumetric measures. 125 ml plastic vessels, each contains 30 ml of culture medium (the composition of culture media depended on the treatment) were cultured with 74 μl of 300–600 μm micro-explants. The vessels were constantly shaken (150 rev min^{-1}) during culture at 20 °C and exposed to 16 h photoperiod (40 μ mol m^{-2} s^{-1} light intensity). Micro-explants developed to microshoots in culture. The number of developing microshoots and their viability, assessed as total microshoot fresh weight, were evaluated after 21 days of culture.

2.2. The effect of PPM on microshoot development

2.2.1. Experiment 1

Three concentrations of PPM (1, 2, 3 mL L^{-1}) (Apollo Scientific Limited, UK) were used with both, plant growth regulator (PGR) free S23 liquid medium and S23 supplemented with 9.29 μM kinetin and 4.92 μM IBA. Five culture vessels were used with each treatment. The aim of this experiment was to investigate the effect of using relatively high concentrations of PPM on the growth of microshoots and to determine if the PPM has any effect on the induction of micro-explants or their subsequent development.

2.2.2. Experiment 2

Four concentrations of PPM (0, 0.25, 0.5, 1 mL L^{-1}) were added to S23 liquid medium used during blending and the micro-explants obtained were cultured in S23 supplemented with 9.29 μM kinetin and 4.92 μM IBA supplemented with the same four different concentrations of PPM (0, 0.25, 0.5, 1 mL L^{-1}). The aim of this experiment was to investigate the impact of PPM on contamination and whether the presence of PPM in the blending stage is enough to control the contamination in the later stages of culture or whether it is necessary for PPM to also be used with the culture media to control the contamination. Five culture vessels were used with each treatment.

2.3. The effects of PPM on the conversion rate and viability of artificial seeds of cauliflower

Eleven day-old microshoots produced using the 212–300 μm size class explants and cultivated in S23 culture medium supplemented with 9.29 μM kinetin and 4.92 μM IBA were used for the production of artificial seeds. Sodium alginate was sterilized by tyndallization which included four main steps described as follow: (1) heat at 80 °C for 15 min; (2) rest at room temperature for 5 h; (3) heat at 90 °C for 15 min; (4) rest overnight and heat at 90 °C for 15 min. Microshoots were mixed with sterilized sodium alginate 2% (w/v) and dropped into a sterilized (autoclaved) solution of calcium chloride 15 g L^{-1} (102.03 mM) using a sterilized pipette. Microshoots were left in the calcium chloride for 30 min for full polymerization of the beads produced. The artificial seeds were then transferred to maintenance S23 for 30 min followed by a quick wash with sterile distilled water.

Artificial seeds were then cultivated on four different sterilized substrates (compost, vermiculite, perlite and sand) contained in plastic cuboid culture vessels (10 cm × 10 cm × 8 cm). Each vessel used contained 200 ml of the substrate described. Four different irrigation solutions were assessed with each culture substrate as follows: (1) S23 supplemented with 1 mL L^{-1} PPM; (2) S23 supplemented with 5 mL L^{-1} PPM; (3) S23 supplemented with 10 mL L^{-1}

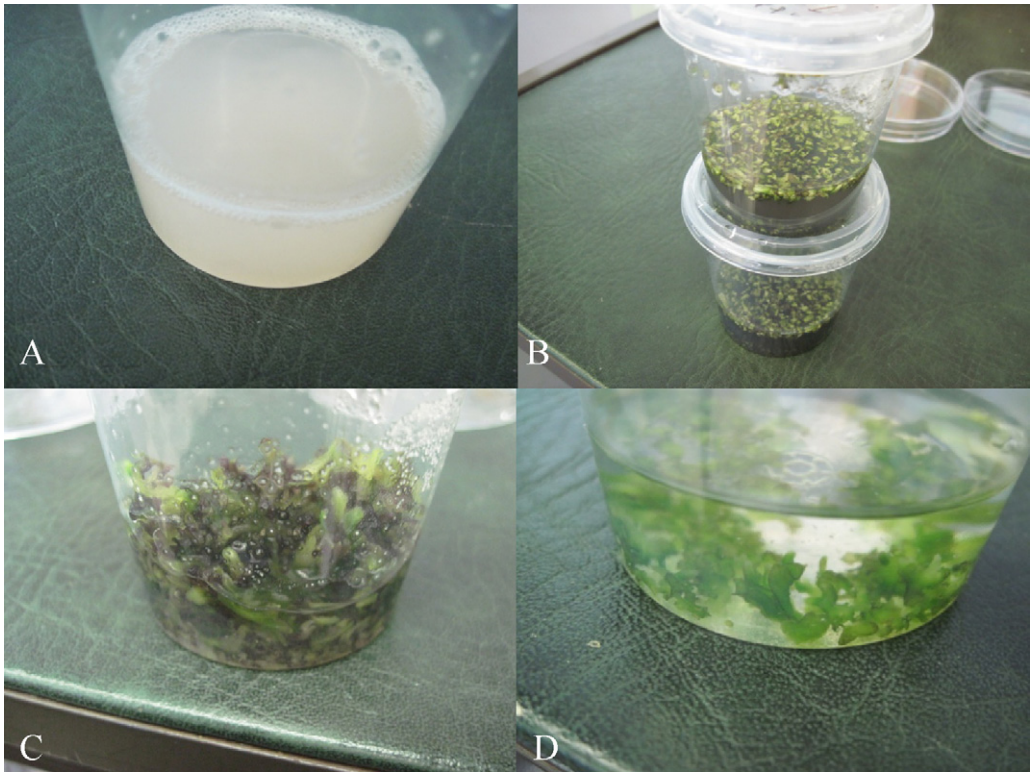

Fig. 1. Photographs of typical cultures after 21 days: (A) PPM-free culture medium. (B) 0.25 PPM mL L^{-1}. (C) 0.5 mL L^{-1} of PPM. (D) 1 mL L^{-1}.

PPM; (4) S23 with no PPM. Each vessel was irrigated with 75 mL of the irrigation solution and the vessels were covered and kept in a growth cabinet (Sanyo, Leicestershire, UK) at 23 °C. The aim of this experiment was to assess the commercial substrates suitability for the conversion and establishment of artificial seeds and the effect of using high concentration of PPM on the development of artificial seeds. Artificial seeds were cultivated in 5 lines of 6 seeds and each line was considered as a replicate. Artificial seed conversion rate and viability was assessed as fresh weights of plantlets produced after 50 days of culture.

2.4. Statistical analysis

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 using the least significant difference test (LSD) at 5% level of probability.

3. Results

3.1. The effect of PPM on microshoots development

Plant growth regulators (PGR) were essential for explants to develop and no explants developed in PGR free culture media regardless of the concentration of PPM used. The use of relatively high concentrations of PPM had a damaging effect on the plant material and none of the explants grew using concentrations higher than 1 mL L^{-1}. The average number of developing microshoots using 1 mL L^{-1} PPM was found to be (36.166 ± 8.21) microshoots per vessel with average weight of (0.313 ± 0.034) g.

The addition of PPM to the blending medium did not control the contamination even when it was used at relatively high concentration (1 mL L^{-1}) and contamination killed all the micro-explants in the PPM-free culture medium (Fig. 1A). Thus, the presence of PPM in the culture medium was found to be an essential requirement irrespective of the concentration of PPM used with the blending medium (Figs. 2 and 3).

It was observed that the higher the concentration of PPM used with the culture medium, the lower the number of explants developed. The use of a relatively low concentration of PPM (0.25) mL L^{-1} was found to be the optimal in terms of the number of developing microshoots, although their subsequent growth, as assessed as average weight was significantly lower ($P < 0.001$). It was also noted that there were color differences associated with the PPM levels. Low concentrations (0.25 mL L^{-1}) of PPM led to explants showing a high level of darkness and a consequent browning of the culture medium probably caused by the accumulation of phenol-like compounds (Fig. 1B) and could be the reason why the microshoots stopped growing. Microshoots growing in media supplemented

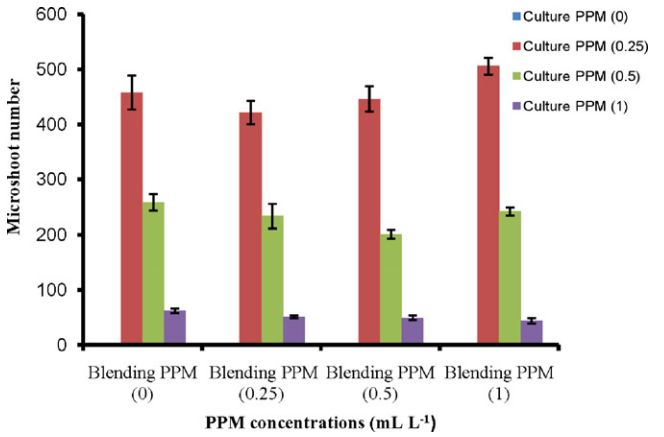

Fig. 2. The effect of PPM used with culture and blending media on the number of developing microshoots after 21 days culture days (LSD = 16.22 for both of blending PPM and culture PPM; LSD = 32.44 for the interaction between culture and blending PPM).

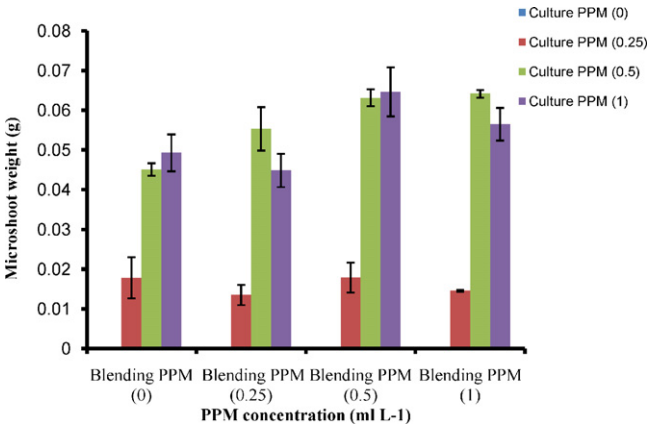

Fig. 3. The effect of PPM used with culture and blending media on the average weight of microshoots after 21 days culture (LSD = 0.003 for both of blending PPM and culture PPM; LSD = 0.0078 for the interaction between culture and blending PPM).

with 1 mL L^{-1} PPM were lighter in color than those produced using 0.5 mL L^{-1} PPM after 21 days of culture (Fig. 1C and D). The difference in color could be attributed to the differences in the density of developing microshoots which affects relative nutrient supply. The use of 0.5 mL L^{-1} PPM added to the culture medium and 1 mL L^{-1} PPM with the blending medium was found to be most effective to control the contamination in the culture system without a significant reduction in the optimal development of the microshoots (Figs. 2 and 3).

3.2. *The effects of using commercial substrates (compost, perlite, vermiculite and sand) and the use of PPM in the fertigation on artificial seed conversion rate and viability*

High significant effects of the culture substrates were observed in terms of artificial seed conversion rate and viability ($P < 0.001$). The use of sand was found to be optimal with a conversion rate of 91.7% and a plantlet weight of 0.1263 g.

Significant differences were also observed when PPM was used with liquid fertigation in terms of the conversion rate ($P < 0.001$) and viability ($P = 0.037$) (Fig. 4). While the use of PPM at relatively low concentration (5 mL L^{-1}) had a negative effect reducing the conversion rate of artificial seeds, the presence of PPM at relatively high concentration did not have this effect (Fig. 4). In terms of

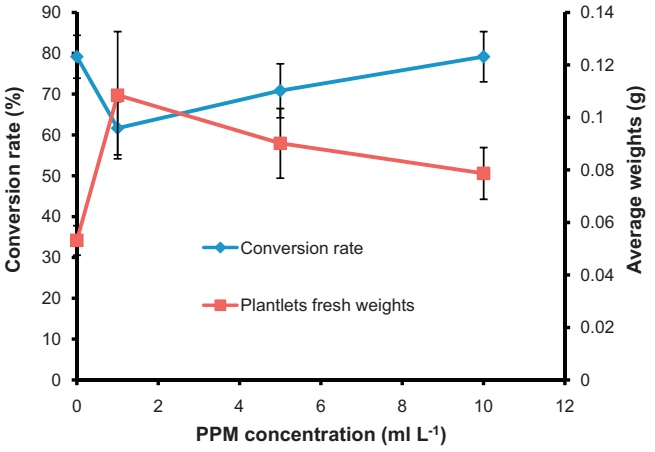

Fig. 4. The effect of using PPM in the medium used for irrigation on artificial seed viability and conversion rate (LSD = 8.333 for conversion rate; LSD = 0.0377 for plantlet fresh weights).

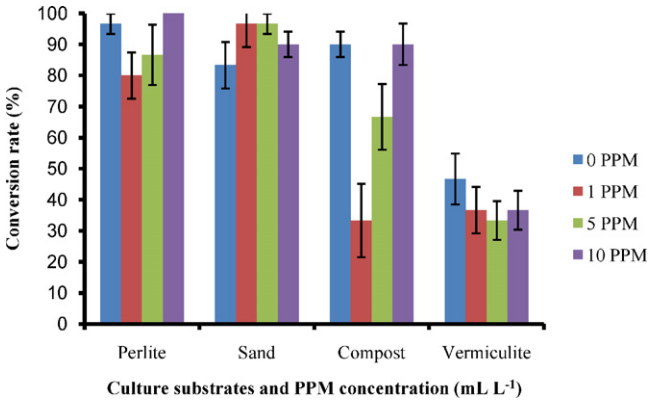

Fig. 5. The effects of PPM, culture substrate and the interaction between culture substances and PPM on the artificial seed conversion rate (LSD for culture substrates and PPM = 6.95; LSD for the interaction between culture substrate and PPM = 13.91).

subsequent development of the artificial seeds, the use of PPM had a positive overall effect. However, the higher the PPM concentration used, the lower the observed artificial seed viability (Fig. 4).

A high interaction between culture substrates and PPM was observed ($P < 0.001$) in terms of conversion rate (Fig. 5). High conversion rates were obtained using different treatments (Fig. 5) like the use of relatively high concentrations of PPM (10 mL L^{-1}) with perlite or the use of relatively low concentrations of PPM with sand (Fig. 5). However, the use of relatively high concentration of PPM (10 mL L^{-1}) with perlite led to a high reduction of artificial seeds viability (Fig. 6). Therefore, the use of PPM at relatively low concentration with the use of sand was recommended as this treatment gave the optimal results in terms of artificial seed conversion rate and viability at high significant difference compared with the use of other treatments ($P < 0.001$) (Fig. 7).

4. Discussion

An inverse relationship between PPM concentration and the number of explants that developed in the liquid culture medium was observed in this study. This is in agreement with Compton and Koch (2001) who found that the percentage of melon cotyledon explants which formed callus and somatic embryos and furthermore the number of embryos per explant were decreased when incubated in embryo initiation and embryo development media containing more than 5 mL L^{-1} PPM. Also Niedz (1998) mentioned that PPM does not affect the viability of sweet orange (*C. sinensis* L.)

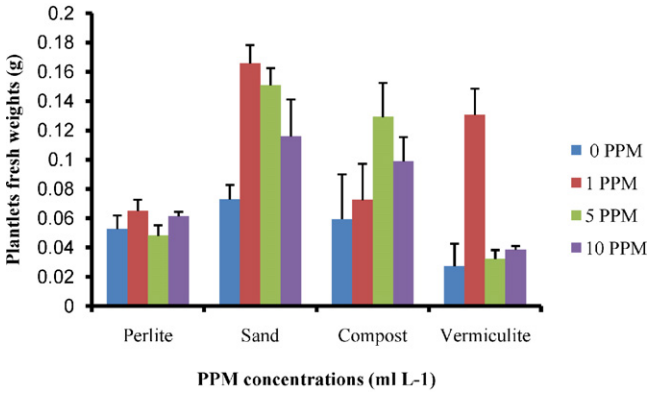

Fig. 6. The effects of PPM, culture substrate and the interaction between culture substances and PPM on the artificial seed viability assessed as plantlet average weights (g) (LSD for culture substrates and PPM = 0.031; LSD for the interaction between culture substrate and PPM = 0.059).

Fig. 7. Plantlets obtained using sand and S23 medium after 50 days of culture.

explants and does not reduce their growth ability when it is used in concentrations lower than 1.0 mL L^{-1}. Kraj and Dolnicki (2003) indicated that there was no negative effect of PPM upon the growth and development of callus in European beech (*Fagus sylvatica* L.). It seems that the determination of the optimal PPM concentration for controlling the contamination without affecting the growth capacity in the culture medium depends primarily on the plant species. For cauliflower microshoots, a relatively low concentration of PPM (0.25 mL L^{-1}) was found to control the contamination effectively and led to the production of the highest number of developing explants. Nevertheless, the viability of these developing explants was subsequently limited by the high level of phenol-like components produced. The numerous numbers of growing explants produced by the blending treatment could be the cause of the high level of phenol-like compounds as competition has an early onset and exhaustion of essential media ingredients can lead to the production of toxic biochemicals which leach into the medium. The darkening of the culture medium caused by these compounds is considered to be a serious problem in tissue culture systems (Ozyigit, 2008; Titov et al., 2006).

It seems that there is a relationship between phenolic-like component exudation and the concentration of PPM used since there were no signs of the presence of these compounds when PPM concentrations were higher than 0.25 ml L^{-1}. This accords with Chamandoosti (2010) who mentioned that there is a relationship between chemical composition of the medium and phenolic production. However, the large number of developing explants and the fast rate of growth using low concentrations of PPM led to an accumulation of released phenolic-like components since PPM might work as growth retardant in this case, limiting the number and the speed of explant growth.

Gupta et al. (2000), Kumria et al. (2003) and Ozyigit (2008) suggested that the age of the mother plant and the explants are the most efficient factors for controlling the total amount of phenolic compounds in the medium. Chamandoosti (2010) found that the composition of medium and type of explants are the most important factors for decreasing the phenolic compounds in the culture medium. Mante and Tepper (1983) reported that the browning caused by phenolic compounds in the culture medium can be prevented using a mixture of the antioxidants, i.e. ascorbic acid, citric acid and cysteine. These suggestions, to reduce the amount of phenolic compounds produced and to improve the quality of microshoots (viability), require further evaluation with cauliflower.

This study demonstrated a high conversion capacity of cauliflower artificial seeds in sterilized commercial substrates (compost, vermiculite, perlite and sand). Significant effects of PPM and the interaction with culture substrates were observed on both conversion rate and subsequent viability of artificial seeds. This could be attributed to the effect of substrate physical structure or of cation exchange capacity of the substrates. PPM seems to have a role not just in the control of contamination in the culture substrate but also on the development and growth of artificial seeds since Rihan et al. (2011) indicated that the optimal substrate for cauliflower artificial seeds growth was compost when S23 supplemented with PGRs were used as irrigation solutions. Many studies have investigated the possibilities of sowing artificial seeds in soil or commercial substrates, reporting, for example, on the use of vermiculte, sand and soil for the cultivation of mulberry artificial seeds by Machii and Yamanouchi (1993) and the use of soil for alfalfa artificial seed conversion substrate by Fujii et al. (1989). The positive effects of using PPM were also reported by Lata et al. (2009) who indicated that the addition of PPM had a positive result on overall seedling growth and a remarkable improvement in the regrowth and conversion rate of *Cannabis sativa* L. artificial seeds.

5. Conclusion

This study showed that PPM can be an effective agent for controlling the contamination in the cauliflower microshoot micropropagation system. It was observed that the determination of the suitable concentration is an essential requirement for using PPM since the high concentrations can be harmful to plant materials. The use of 0.5 mL L^{-1} PPM added to the culture medium and 1 mL L^{-1} PPM in the blending medium was found to be optimal for controlling the contamination without causing a reduction in the effectiveness of the culture system. This treatment is recommended for use when microshoot quality is a priority since a huge number can be produced from each curd using the protocol described and applying the described treatment. Lower concentrations of PPM increased the number but reduced the viability of microshoots produced.

It was clear that the effect of PPM on artificial seed conversion rate and viability depends on the both PPM concentration and the culture substrate used. When low concentrations of PPM irrigation solution were used with sand as a culture substrate then both the conversion rate and viability of artificial seeds were good. This finding prepares the way forward for using cauliflower artificial seeds in vivo.

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