Artificial Seed Production from Encapsulated Microshoots of Cauliflower (*Brassica oleraceae* var. *botrytis*)

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Abstract

A cost effective protocol for the production of cauliflower microshoots suitable for encapsulation was designed. Microshoots were encapsulated in sodium chloride matrices. The use of 2% of sodium alginate and 15 g/L of dehydrate calcium chloride produced the optimal quality of artificial seeds (rigidity, conversion rate and viability). Of the various plant growth regulator combinations used with the microshoot liquid culture medium, the use of 1 mg/L of IBA (indole butyric acid) and 1 mg/L Kinetin was found to be optimal in terms of the conversion rate and viability of artificial seeds. To standardize a medium composition of artificial endosperm of synthetic seeds, different concentrations and combinations of plant growth regulators with S23 (4.4 MS + 30 g/L sucrose) medium were used in the beads to achieve optimum conversion rate and viability on an in-vitro medium. Whilst several combinations of plant growth regulators gave a conversion rate up to 100% (for example (0.5 mg/L Kinetin + 0.5 mg/L IBA), (1 mg/L Kinetin + 0.5 mg/L NAA (naphthaleneacetic acid)) and (1 mg/L Kinetin + 1 mg/L IAA (indole-3-acetic acid)), no significant effect on the viability of artificial seeds was found when these combinations were used. Artificial seeds were cultivated in a semi-solid medium containing several types and concentrations of auxin, 2 mg/L of IBA gave the best results in terms of artificial seed viability. However, artificial seed conversion rate was not significantly affected by the auxins and full conversion rate was obtained using many different treatments. This research indicated the feasibility of using artificial seeds as a promising alternative to seeds produced by traditional methodology.

INTRODUCTION

Several studies have investigated the capacity of applying artificial seed technique to many kinds of plants. Many explants types have been encapsulated for the production of artificial seeds, for example, the use of shoot buds derived from lam (*Progonatherum paniceum*) calli (Wang et al., 2007), node explants of mugwort (*Artemisia vulgaris*) (Sujatha and Kumari, 2008), shoot tips of *Rauvolfia serpentine* (Ray and Bhattacharya, 2008), somatic embryos of guava (*Psiduim guajava* L.) (Rai et al., 2008), somatic embryos from mature seeds of rauli-beech (*Nothofagus alpine*) (Cartes et al., 2009) and axilliary buds of hemp (*Cannabis sativa* L.) (Lata et al., 2009).

In the cauliflower, several protocols have been investigated to produce artificial seeds through encapsulated somatic embryos (Fransz et al., 1993; Redenbaugh et al., 1986). However, these authors emphasized the difficulty faced in producing artificial seeds 'en masse'. This study aimed to optimize a full protocol for the production of cauliflower artificial seeds by the encapsulation of microshoots derived from curd meristematic tissues.

MATERIAL AND METHODS

Two cultivars of cauliflower, 'Dionis' and 'Mascaret', were obtained from a 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. The use of 2 cultivars gave a continuous supply of cauliflower heads over the experimental period.

Large pieces (1-5 cm) of curds were surface 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 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 meristematic clusters were then homogenized using a commercial blender (Waring model 800) at approximately 1700 rev/min in maintenance S23 liquid medium (MS 4.4 g/L + sucrose 30 g/L) for 30 s. The liquid containing the microexplants was then sieved through precision sieves (212, 300 and 600 μm) (Endecotts Ltd., London, UK). The microexplants were collected off the sieves, weighed and converted to aliquots of explants using small precision volumetric measures (74 or 240 ± 2 μl). 100 ml containers, each containing 30 ml S23 medium supplemented with 2 mg/L Kinetin and 1 mg/L IBA, were cultured with a constant volume of explants (74 μl). The pots were constantly shaken (150 rev/min) during culture at 20°C and exposed to 16 h photoperiod for 12 days. Microshoots produced were mixed with a sterile solution of sodium alginate containing S23 (4.4 g/L MS + 30 g/L sucrose) and supplemented with 1 ml/L PPM (plant preservative mixture) to control the contamination. Different concentrations of sodium alginate (1, 2, 3, 4, 5% (w/v)) (Sigma Ltd.) were investigated. The microshoots contained in the sodium alginate solution were dropped individually into a 10 g/L (68 mM) concentration of sterile $CaCl_2.2H_2O$ solution and were left for 30 min and then transferred to S23 media for 30 min followed by a quick wash with sterile distilled water. The artificial seeds produced were dispatched on a maintenance semi-solid S23 (4.4 g/L MS + 3% sucrose + 7 g/L agar) supplemented with 1 ml/L PPM and 2 mg/L IBA. The artificial seeds were kept at 23°C and 16 hours photoperiod provided by fluorescent lighting (150 μmol mol m^2). Applying the same system but with 2% sodium alginate solution, the effect of three concentrations of $CaCl_2.2H_2O$ (5, 10 and 15 g/L) (34, 68 and100 mM) was evaluated. The effects of the sodium alginate and $CaCl_2.2H_2O$ concentrations on the conversion rate and the viability of artificial seeds assessed as average plantlet fresh weights evaluated after two weeks of culture. For each treatment, three plastic culture vessels (10×10×8 cm), each containing 100 ml of maintenance S23 media supplemented with 1 ml/L PPM and 2 mg/L of IBA were used. Twelve artificial seeds were cultivated in each container and each container was considered as a replication.

The effect of plant growth regulator combinations used in the liquid culture medium for the production of microshoots on their suitability for artificial seed production was investigated. This experiment was divided in two steps. Firstly, maintenance S23 was supplemented with nine combinations of Kinetin (1 and 2 mg/L) and IBA or NAA (1 and 2 mg/L). Secondly, S23 was supplemented with four combinations of Kinetin (1 and 2 mg/L) and IAA (1 and 2 mg/L) and was compared with the use of S23 supplemented with 2 mg/L of Kinetin and 1 mg/L of IBA. Artificial seed conversion rate and their viability was assessed as the number and fresh weight of plantlets evaluated after 20 days of culture in semisolid medium supplemented with 2 mg/L IBA. Three replications, each consisting of 10 artificial seeds, were used for each treatment. Each ten artificial seeds were cultivated in plastic culture vessels. The treatments were distributed randomly within the vessels.

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

2% sodium alginate concentration was found to be optimal, producing spherical beads of 5-7 mm diameter. The viscosity of sodium alginate solution was increased with higher concentration producing drop-shaped bead and concentrations lower than 2% gave poor quality small beads. Encapsulated micro-shoots placed in a growth chamber germinated within a few days whatever the initial sodium alginate concentration used (1-5%). Thus, it was concluded that there was no toxic effect of sodium alginate on the plant tissues. There was also no effect of sodium alginate concentration on the viability of produced artificial seeds since there was no significant difference between the average weights of plantlets obtained after two weeks of culture (P=0.568). However, while all encapsulated micro-shoots showed the ability to convert regardless of the initial concentration of $CaCl_2.2H_2O$ used, a significant difference was observed in terms of the fresh weights of plantlets obtained after two weeks of culture. The concentration of 15 g/L $CaCl_2.2H_2O$ was found to produce the highest plantlet average weights (P=0.022) in comparison with the use of other $CaCl_2.2H_2O$ concentrations (Fig. 1).

The Effect of the Liquid Media Plant Growth Regulators on the Suitability of Microshoots for Artificial Seed Production

It was observed that the Kinetin concentration had a significant effect on the conversion rate of artificial seeds produced (Table 1) and the use of the relatively lower concentration 1 mg/L gave the optimal results at conversion and was assessed to be 96.7%.

It was also observed that the concentrations of auxin had a significant effect (Table 1) and the use of 1 mg/L of auxin was found to be better than the use of 2 mg/L. The conversion rates were found to be 97.5 and 89.17% at 1 and 2 mg/L auxin concentrations respectively.

A very high interaction between the Kinetin concentrations and auxin types was found ($P<0.001$). Using 1 mg/L Kinetin with IBA auxin type, the conversion rate was assessed at 100%.

A very significant interaction was also observed between Kinetin concentration, auxin type and auxin concentration (Table 1). It was found that the use of (1 mg/L Kinetin + 1 mg/L IBA), (2 mg/L Kinetin + 2 mg/L NAA), (1 mg/L Kinetin + 1 mg/L NAA) and (1 mg/L Kinetin + 2 mg/L IBA), gave the best results (100% conversion rate) and it was observed that there was no significant difference between these treatments and the use of (2 mg/L Kinetin + 1 mg/L IBA) which was considered as a standard used in the next part of this experiment for the comparison with other plant growth regulator combinations (Fig. 2).

The Effect of Plant Growth Regulator Combinations on the Artificial Seed Viability

Auxin concentration had a significant effect on the viability of artificial seeds (Table 2) with 1 mg/L of auxin concentration giving the highest weight 1.111 g of plantlets produced compared to 2 mg/L giving plantlet average weight of 0.490 g.

A highly significant interaction was found between the Kinetin concentration and auxin type used with the microshoot culture medium on the subsequent viability of artificial seeds ($P<0.001$). It was found that optimal results were produced using either a low concentration Kinetin with IBA or a high concentration of Kinetin with NAA.

A significant correlation between Kinetin concentration, auxin types and auxin concentrations was also observed (Table 2). However, the best plant growth regulator combination was obtained using 1 mg/L Kinetin + 1 mg/L IBA and 2 mg/L Kinetin + 1 mg/L NAA treatments (Fig. 2). It was found that there was a highly significant difference between these treatments and the use of 2 mg/L Kinetin + 1 mg/L IBA which was used as a standard in the next experiment.

In the second part of this experiment, it was observed that the use of 2 mg/L of Kinetin with 1 mg/L of IBA was found to be better than all the combinations of Kinetin with IAA in terms of both artificial seeds conversion rate and viability.

Overall in this experiment the use of 1 mg/L of Kinetin incorporated with 1 mg/L of IBA as a plant growth regulator combination was found to be the optimal in terms of artificial seed conversion rate and viability.

DISCUSSION

Sodium alginate has been extensively used for artificial seed production in different species (Ara et al., 2000; Rai et al., 2009; Redenbaugh et al., 1987). The use of 2% (w/v) sodium alginate was found to be optimal producing 5-7 mm diameter cauliflower artificial seeds and allowing the easy emergence of shoot and root from the beads and the use of 15 g/L (100 mM) dehydrate calcium chlorite was found to be the optimal and more effective than using relatively lower concentrations. The mixing of 2% of sodium alginate with 100 mM of $CaCl_2.2H_2O$ for 20-30 min has been reported to be suitable for bead formation in different species (Ara et al., 2000, 1999; Saiprasad, 2001). The current results contradict reports that higher level of encapsulated somatic embryos conversion was obtained by increasing the concentration of sodium alginate and reducing the concentration and exposure time to calcium chloride (Cartes et al., 2009; Prewein and Wilhelm, 2003). It was reported that the high concentration or the long exposure of the embryos to the calcium chloride results in more absorption and penetration of $CaCl_2$ in the embryo, which can lead to growth inhibition (Redenbaugh et al., 1986). However, this inhibition role of calcium chloride was not observed in the current study.

This study showed that the organization of cauliflower microshoots is highly suitable for encapsulation as artificial seeds. The types and concentrations of plant growth regulators used with microshoots culture media had a great effect on the artificial seed conversion rate and viability. The optimal combination of plant growth regulators used for the production of microshoots suitable for artificial seed production (1 mg/L Kinetin + 1 mg/L IBA) was found to be different from that used for the production of maximum number with highest average weight of microshoots produced in liquid media (2 mg/L Kinetin + 1 mg/L IBA) (data not shown). Therefore, plant growth regulators used had major effects not only on the number or the fresh weight of developing microshoots but also on the physiological structure of the microshoot produced. The use of a relatively high concentration of Kinetin led to the production of poor quality microshoots which were not compact and which were affected by the encapsulation procedures, reducing the conversion rate and the viability of artificial seed and the use of a relatively low concentration (1 mg/L) was found to be optimal. The type and the concentration of auxins also affected the conversion rate and the viability of artificial seeds. Although several studies have demonstrated the positive effect of auxins on the root formation and development (Blakely et al., 1988; Boerjan et al., 1995; Reed et al., 1998), in the current research, none of the auxin types and concentrations used induced root formation. This could be attributed to the presence of Kinetin in the liquid medium. It has been reported by others that the inclusion of cytokinin decreases the number of lateral roots (Hinchee and Rost, 1986; Nakashimada et al., 1995; Rani Debi et al., 2005). Hinchee and Rost (1986) reported that the auxin:cytokinin ratio has an essential role in co-ordinating lateral root growth in pea seedlings. However, although there was low rooting observed during explant culture in the liquid medium, the microshoots showed the ability to convert after capsulation in the semisolid medium supplemented with 2 mg/L IBA producing proper plantlets.

CONCLUSION

Cauliflower microshoots are suitable for encapsulation as artificial seeds. The use of sodium alginate solution and calcium chloride at 2 and 1.5% concentrations respectively gave the optimal results in terms of shape, rigidity, conversion rate and viability of artificial seeds. Moreover, the quality of microshoots and their suitability for encapsulation was affected with the composition of the culture medium and it was optimized to produce the optimal seed quality.

This study demonstrated that the cauliflower curd has a high capacity for the

production of good quality artificial seeds. The capacity of microshoot production coupled with encapsulation presents a very useful technique for the manipulation of cauliflower germplasm compared to traditional cauliflower tissue culture protocols.

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Tables

Table 1. The effect of microshoot culture plant growth regulators on the conversion rate of artificial seed.

Source	*P*	Sig
Kinetin concentration	0.019	*
Auxin type	0.075	-
Auxin concentration	0.004	**
Kinetin concentration × auxin type	0.000	***
Kinetin concentration × auxin concentration	0.546	-
Auxin type × auxin concentration	0.231	-
Kinetin concentration × auxin type × auxin concentration	0.001	***

Table 2. The effect of microshoot culture plant growth regulators on the viability of artificial seeds.

Source	*P*	Sig
Kinetin concentration	0.468	-
Auxin type	0.513	-
Auxin concentration	0.000	***
Kinetin concentration × auxin type	0.000	***
Kinetin concentration × auxin concentration	0.831	-
Auxin type × auxin concentration	0.599	-
Kinetine concentration × auxin type × auxin concentration	0.000	***

Figures

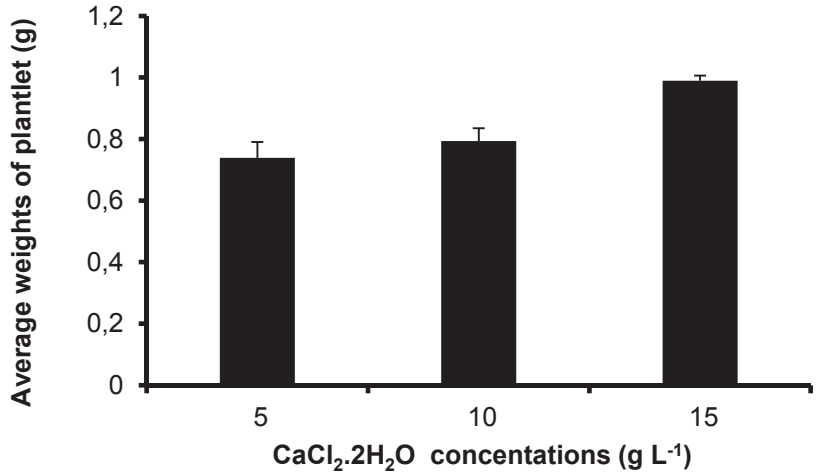

Fig. 1. The effect of the $CaCl_2.2H_2O$ concentrations on artificial seeds viability.

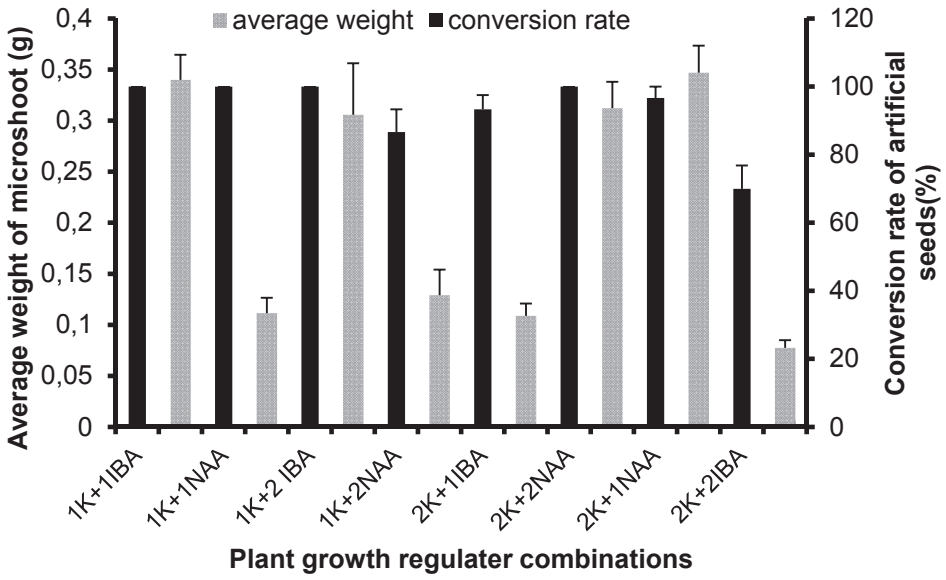

Fig. 2. The effect of the plant growth regulator combinations on the artificial seeds conversion rate (LSD=7.498) and viability (LSD=0.075).