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Effect of storage conditions on conservation of sorghum shoot tips in calcium alginate beads

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Summary
Sorghum artificial seeds were obtained through encapsulation of somatic embryos or adventitious buds in calcium alginate beads. In this paper, the effect of storage conditions on viability of encapsulated plant materials was studied. To obtain plant material for encapsulation, axillary and adventitious buds without shoot development were obtained on seedling shoot tips cultured on MS medium supplemented with 2mg/l BAP and 0.5 mg/l 2,4-D under light condition (100 µmol $m^{-2}s^{-1}$). Subculture of the obtained shoot tips in two weeks intervals resulted in the formation of about 759,375 shoot tips/explants in three months. At the same period, shoot explants cultured on MS medium supplemented with 3 mg/l 2,4-D resulted in the formation of about 270 somatic embryos including embryo maturation in four weeks on MS medium supplemented with 0.56 mg/l BAP and 0.1 mg/l NAA. In sorghum, where artificial seeds can be obtained using of in vitro derived buds or embryos, buds were recommended due to feasibility to obtain high number of plant materials with good quality in short time, the essential need for mass production of artificial seeds. Storage of the encapsulated plant materials in liquid MS medium supplemented with 4 mg/l BAP and 0.5 mg/l 2,4-D in laboratory refrigerator was recommended, where the encapsulated plant materials conserved its potential to convert. Isenzyme studies indicated that alginate matrix exerted slight changes in isozyme expression in encapsulated plant materials.

Key words: Artificial seeds, calcium alginate, isoenzymes, sorghum, tissue culture.

Introduction:

Artificial seed technology represents an attractive alternative to traditional micro propagation for production and delivery of clonal plantlets, but several aspects of the technique are still underdevelopment and hinder its commercial application (Brischia et al. 2002).

Construction of artificial seeds has been done by encapsulating explants within calcium alginate beads to protect them from dehydration and mechanical damage that may occur during preservation, transport and sowing. The alginate matrix can be supplemented with nutritive substances and growth regulators (artificial endosperm), which is necessary for preservation and re-growth after sowing. The artificial endosperm may also be supplement with protective substances such as bactericides, fungicides and pesticides to protect the sowing seeds from insects and microorganisms. The components of the artificial seed could be covered with a synthetic seed coat to enable mechanical handling and planting. Machine competent explants have been also frequently used for production of artificial seeds in large scale (Brischia et al. 2002).

In the last two decades, preservation of plant tissue in calcium alginate beads has been increasingly used to produce artificial seeds. There are several potential advantages of this artificial seed technology: (1) ease of handling, (2) low production cost, (3) ease of exchange of plant materials between different laboratories in different counties, (4) genetic uniformity of propagated plants (5) direct delivery to the soil, (6) shorten the breeding cycle, and (7) reduction of the storage space

(Van den houwe et al. 1995; Maruyama et al. 1977; Ganapathi et al. 2001). An additional advantage of the use of artificial seeds is that storage of alginate-encapsulated germplasm at room temperature for several years can be used as alternative tool for conservation under ultra low temperature condition (cryopreservation in liquid nitrogen), which it requires sophisticated techniques and is not always available in developing countries (Maruyama et al. 1997).

The most important obstacle that counteracts the application of artificial seeds at large scale is the re-growth of encapsulated plant materials after sowing especially under non-aseptic conditions. Therefore, storage conditions of artificial seeds before sowing were found to be essential factors to enhance the plant regeneration rate even under non-aseptic conditions (Maruyama et al. 1997). Storage of artificial seeds containing somatic embryos or non-embryogenic somatic propagules has been attempted in a few plant species with various degree of success (Maruyama et al. 1997, Pattnaik and Chand 2000).

Encapsulation of *in vitro*-derived vegetative propagules such as axillary buds or shoot tip meristems could be used for mass clonal propagation of high value crops at reasonable cost, especially when viable somatic embryos are not easily obtained (Pattnaik and Chand 2000). In sorghum, regeneration via adventitious buds or somatic embryos was reported (Hassanein et al. 2008), but for the production of artificial seeds, fast regeneration protocols to decrease somaclonal variation and high rate of multiplication to obtain enough plant materials were essential perquisite for the success of this approach. Consequently, the aim of this work is to establish a protocol for mass artificial seeds production in sorghum. Another aim is to know the necessary storage conditions, which conserve the viability of encapsulated plant materials during artificial seed storage.

Materials and Methods:

Preparation of plant materials:

Sorghum seeds were surface-sterilized in 5% commercial bleach solution for 10 min followed by 5 min rinse in 75% ethanol. After three successive rinses in sterile distilled water for 5 min each, the seeds were placed on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, without growth regulators, for seed germination. The medium was solidified with 8 g agar at pH 5.8. Vitamins (mgl^{-1}) were: myo-inositol (100), vitamin B1-hydrochloride (4), nicotinic acid (4), pyridoxal hydrochloride (0.7), biotin (0.04) and colic acid (0.5). The seeds were germinated at 25 ± 2^0C with 16-h photoperiod (100 μmol $m^{-2}s^{-1}$). A three mm section of seedlings containing shoot apical meristem, leaf primordial and a portion of mesocotyl coleoptile was cut. These cuttings were cultured on MS medium supplemented with 0.5 mg/l 2,4-D and 2 mg/l BAP (multiplication medium) as was described by Zhang et al. (1998). Fifteen explants were placed on multiplication medium or on MS medium supplemented with 3 mg/l 2,4-D for induction of somatic embryo formation. After seven days the leaves, coleoptile and elongated stem were removed from each explant and cultured on the same medium. This process was repeated until the bud multiplication or embryogenic mass was detected on each explant.

Induction of shoot tip multiplication for synseed production:

Shoot tip multiplication on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l 2,4-D resulted in the formation of bud clumps on each shoot tip explant in four weeks. Bud clumps were gently separated into individual bud or compact clump with two or three buds and transferred to new multiplication medium for further two weeks. Bud progeny after the fifth subculture was used for encapsulation purpose.

Induction of somatic embryo formation for synseed production:

Induction of friable embryogenic masses formation was obtained when the shoot tips were incubated on MS medium containing 3 mg/l 2,4-D under dark condition and subcultured every two weeks interval. In ten weeks, valuable masses of friable callus were obtained. The obtained masses were sub-cultured on MS medium supplemented with 0.1 mg/l NAA and 0.56 mg/l for four weeks under light condition (16-h, 100 μmol $m^{-2}s^{-1}$) for embryo maturation. The mature embryos embedded in embryogenic masses were picked, cleaned and subjected for encapsulation processes.

Encapsulation of shoot tips and somatic embryos:

Sodium alginate (gel matrix) was added at a concentration of 3.5% to liquid multiplication medium. For complexion 75 mM CaCl2 solution (complexion agent) was prepared using multiplication medium too. When embryos were used to form synseeds, the multiplication medium was substituted by embryo maturation medium. Both the gel matrix and complexion agent were autoclaved at 120^{o}C for 20 min and stored at room temperature. Encapsulating was accomplished by dipping the shoot tips or cleaned somatic embryos with forceps in gel matrix, followed by dropping into the complexion agent for 30 min. To wash away calcium chloride residues, alginate beads containing shoot tips or embryos were collected and rinsed twice for 10 min each in multiplication or embryo maturation medium, respectively. The obtained beads thus were 5-6 mm in diameter.

Effect of encapsulation procedure and calcium alginate matrix on viability of encapsulated plant materials

To study the effect of encapsulation procedure and alginate matrix on viability and re-growth of encapsulated plant materials, the encapsulated and un-encapsulated plant materials were transferred directly after their formation on conversion substrate (MS basal medium). They were incubated in glass jars containing 25 ml of conversion medium under tissue culture condition. Viability and conversion of the synseeds after 4 weeks were estimated.

Effect of conservation medium on viability and conversion of synseeds:

A set of encapsulated shoot tips were conserved in closed glass jars in different substratum (Table 1). Each jar contained 30 ml substratum, and stored in laboratory refrigerator. A group of un-encapsulated shoot tips or somatic embryos were stored under the above condition to serve as a control. Viability of shoot tips was tested weekly for four weeks. The shoot tips were considered alive if the shoot tips were still green with no necrosis. Viable seeds were transferred to convert on MS basal medium.

Electrophoresis studies:

Two types of plant materials were used in this study to investigate the changes in gene expression under the influence of encapsulation of shoot tips in calcium alginate beads. Half gram of cultured materials was ground at 4^{0}C in a mortar in 0.5 ml of extraction buffer that consisted of 0.1 μM tris-HCl, pH 7.0 and 0.002 M cysteine. The homogenate was centrifuged at 15,000 rpm at 4^{0}C for 15 min. Supernatants were collected for immediate electrophoresis in 7.5% polyacrylamide slab gels. Gels were run at 18 mA for 6 h at 10^{0}C in 0.025 M tris + 0.192 M glycine buffer (pH 8.9). Peroxidase and indophenol oxidase were stained according the protocols of Siegel and Galston (1976) and Brewer (1970).

Results and discussion:

Fast multiplication of axillary and adventitious buds without shoot development was induced when shoot tips were cultured on MS medium supplemented with 2mg/l BAP and 0.5 mg/l 2,4-D under light condition. In four weeks, clusters of 15 buds/explant were obtained. Subculture of the obtained shoot tips in two weeks intervals resulted in the formation of about 759,375 shoot tips/explant in three months (5 subcultures). On the other side, shoot explants cultured on MS medium

supplemented with 3 mg/2,4-D resulted in the formation of friable embryogenic masses (in 10 weeks) and the formation of about 270 somatic embryos after further four weeks on maturation medium (MS with 0.1 mg/l NAA and 0.56 mg/l). Since artificial seeds can be obtained by encapsulating *in vitro* obtained buds (Fig. 1) or embryos (Fig. 2), use of buds in sorghum may be recommended to produce artificial seeds due to feasibility to obtain high number of plant materials with good quality in short time, that may be regarded as important requirements for mass production of artificial seeds.

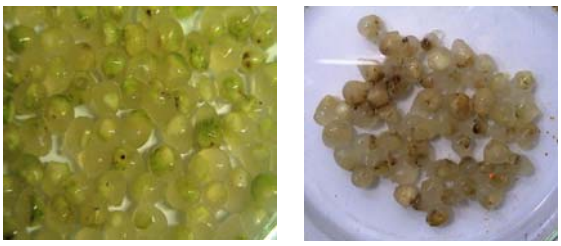

Fig.1: Photographs of encapsulated buds (left) and embryos (right) of sorghum

To study the effect of encapsulation procedure and alginate matrix (3.5% calcium alginate) on the viability of the encapsulated tissues, encapsulated and un-encapsulated shoot tips or embryos were placed, immediately after their preparation, on conversion medium (MS basal medium) and stored under the described conditions. In four weeks, the viability of the un-encapsulated shoot tips was 100%, however the viability of non encapsulated somatic embryos was 83%. On the others side, the viability of encapsulated buds was 98% but it was 65% in case of encapsulated somatic embryo. This indicated that, the frequency of successful conversion from encapsulated embryos was significantly affected by the type of the encapsulated plant materials. The results of the present study encouraged to use shoot tips as the plant material and alginate as the matrix to produce artificial seeds in sorghum using the mentioned procedure. In this respect, it should be mentioned that alginate is the most commonly used material to obtain artificial seeds because it is available in large quantities, inert, non-toxic, cheap and can be easily manipulated (Endress 1994), it has encouraged conversion in several plant species (Redenbaugh et al. 1991). However, explants of some plant species need specific inductive treatment before encapsulation in order to obtain shoot and/or root emission during conversion (Micheli et al. 2002).

In the present study, a procedure for encapsulation of shoot tips and somatic embryos of sorghum in alginate beads after conservation for 4 weeks was described. The encapsulated plant materials were stored at $4^{o}C$ for 4 weeks in conservation medium. Similar conditions were used before to obtain good results (Maruyama et al 1997, Pattnaik and Chand 2000; Ganapathi et al. 2001). The obtained buds maintained their regenerative potential after fragmentation and encapsulation. Also, the obtained sorghum somatic embryos were successfully used to produce artificial seeds. The plant materials produced by the procedures described in this study were also reported by many authors in sorghum and in other plant species (Onay et al. 1996; Sicurani et al. 2001). To avoid genetic variation under the influence of tissue culture conditions, encapsulation of the plant materials was carried out after five subcultures. Suppression of the growth of the encapsulated sorghum shoot tip under the influence of oxygen deficiency and low temperature was established in liquid conservation substrate at laboratory refrigerator. (Brodelius et al. 1982; Ganapathi et al. 2001).

Table 1 shows the results of viability of alginate-encapsulated shoot tips after 4 weeks preservation on different conservation substrates in laboratory refrigerator and after further 4 weeks on conversion medium under tissue culture condition. The best results were obtained when the encapsulated buds were stored in liquid MS medium supplemented with sugar and growth regulators, where the viability of the encapsulated buds was 92%. It also expressed the highest conversion rate (83.3%), the highest shoot number (17 shoots) and the highest shoot length (1.57 cm). These values were reduced when double concentration of growth regulators was used.

In addition, the data indicated that absence of growth regulators or sugars was affected negatively on viability and conversion of the encapsulated plant materials. On the other side, store the alginate beads on tap water substratum expressed the lowest values; they improved when the tap water substratum was supplemented with 3% sucrose.

In this work, capsules produced more than one rooted shoots (Fig. 3), confirming the presence of more than one shoot primordium on each encapsulated explant. Similar results were obtained previously by (Sicurani et al. (2001) and Brischia et al. (2002). Meanwhile multiple shoot development from encapsulated embryos was also detected in banana (Ganapathi et al. 2001).

Fig. 3: Photograph showing steps of sorghum capsule conversion resulting in shoot and root formation in four weeks under light condition.

Malate dehyderogenase showed the reverse effect where the encapsulated plant material expressed lower staining intensity that that of un-encapsulated one (Fig .5). Changes in staining intensities of detected bands give an indication about changes in the activity of the studied isoenzyme (Khavkin and Zabtrodina 1994, Hassanein 1998). Peroxidase involved in several developmental processes in the plant cells including cell division and cell differentiation through its affect on auxin /cytokinin ratio (Booij et al 1993). In this work, peroxidase was influenced more that any other studied isozyme where encapsulated plant materials expressed new band (POX-1) under the influence of alginate matrix, it was accompanied with decreased staining intensity of the detected bands in comparison to those of un-encapsulated plant materials.

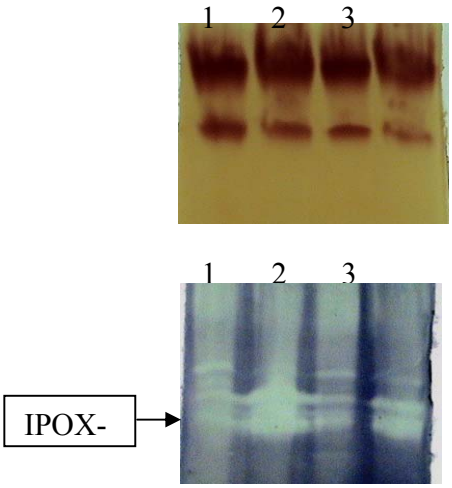

Fig. 4: Native gel electrophoresis of GOT (Top) and IPOX (bottom) isoenzymes in sorghum material cultured on embryo maturation medium for three days under the influence of alginate matrix. Lane 1=buds, lane 2=encapsulated buds cultured on bud multiplication medium, lane 3=embryos, lane 4=encapsulated embryos.

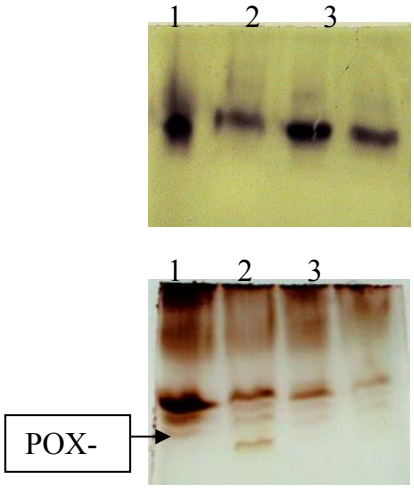

Fig. 5: Native gel electrophoresis of MDH (top) and POX (bottom) isoenzymes in sorghum material cultured on embryo maturation medium for three days under the influence of alginate matrix. Lane 1=buds, lane 2=encapsulated buds cultured on bud multiplication medium, lane 3=embryos, lane 4=encapsulated embryos.

In conclusion electrophoresis studies indicated that calcium alginate matrix exerted relatively low effect on the expression of studied isoenzymes. Plants obtained from artificial seeds of sorghum were hardened off for three weeks and successfully established in an outdoor garden pot with survival frequency of 85%. The obtained plantlets did not show any disorder in their phenotype when they compared with respective donor plants.

Table 1**:** Effect of conservation substrates on viability and conversion of encapsulated buds of sorghum. * Means significantly differed (t-test) from encapsulated buds preserved on MS medium with 3% sucrose, 4 mg/l BAP and 0.5 mg/l 2,4-D at $P < 0.05$ at $4^{o}C$ in laboratory refrigerator. TW = Tap Water, S = Sucrose, GR = 4 mg/l BAP and 0.5 mg/l 2,4-D.

Substrate	Viability	Shoot No./explant	Shoot length (cm)	Conversion frequency
TW	14.06	6*	0.26*	41.00
TW with 3%S	55.5	14*	0.30*	45.00
MS with GR, 3%S	82.00	17	1.57	83.3
MS with 2GR, 3%S	51.26	7*	0.37*	13
MS Without GR	28.33	11*	0.566*	56.5
MS Without GR or 3%S	45.63	13	0.633*	55.5

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