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EVALUATION OF SODIUM ALGINATE AND CALCIUM CHLORIDE ON DEVELOPMENT OF SYNTHETIC SEEDS

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Abstract

The present investigation highlighted the role of sodium alginate (Na-alginate) and calcium chloride ($CaCl_2$) treatments on encapsulation of sugarcane (*Saccaharum officinarum* var. NSG-59) somatic embryos to produce synthetic seeds. Progress in the protocol for direct recovery of plants from man-made seeds under sterile conditions is of major importance. Although it is possible to produce a huge number of plants through tissue culture via embryogenesis or multiple shoot cultures, but their delivery is quite inconvenient. *In vitro* study pertaining to evaluate the efficient synthetic seeds formation from sugarcane somatic embryos was done in Plant Tissue Culture Lab, Department of Botany, University of Sargodha. Encapsulation of somatic embryos was achieved by using Na-alginate (as gelling agent) and $CaCl_2$ (as complexing agent) respectively through dropping method under sterile conditions (inside Laminar air flow cabinet). Different concentrations of Na-alginate (0.5%, 1%, 2%, 2.5%, 3%, 3.5%, and 4%) and $CaCl_2$ (50mM, 70mM, 90mM, 100mM, 150mM and 200mM) were applied in current process.The gelling matrix of 3% Na-alginate and complexing agent of 100mM $CaCl_2$ yielded firm, clear, isodiametric and compact beads formation. It was observed that duration of Na-alginate treatment on bead formation has no effect however, $CaCl_2$ treatment with 100mM Na-alginate for 15 minutes was more appropriate for isodiametric and compact beads formation. The synthetic seeds provide feasible handling, transport and large scale production of viable plants of sugarcane by direct sowing of synthetic seeds in the soil.

Key words: Na-alginate, *Saccaharum officinarum*, Somatic embryos, Encapsulation, Synthetic seeds.

Introduction

Seeds production by using somatic embryos as miniature plant represents the ultimate convenience of crop production when compared with any other form of propagules. Somatic embryos produced *In vitro* can be grown directly into plants under controlled conditions. However, this versatility could be improved further if they are converted into synthetic (artificial) seeds by encapsulating them with synthetic coat and made them analogous to true seeds. Embryos placed individually into the alginate beads would be advantageous for economical mass production of economically important plants and facilitate easy handling and delivery. Encapsulation of somatic embryos with gelling matrix to produce synthetic seeds is modern and advanced technology. However, it would be advantageous to combine the structural and functional efficiency of seedsS with the clonal plants derived via *In vitro* somatic embryogenesis for various other applications.

Synthetic seed technology is headway in conserving endangered plants through encapsulating their propagules under *In vitro* conditions (Naik and Chand, 2006; West *et al*., 2006). Synthetic seeds consisting of coated somatic embryos are distinguished way to spread out influential plant species (Thobunluepop, 2007). A diversity of natural and man-made polymers are on hand for encapsulation, sodium alginate is the most frequently used gel matrix due to its easy gelling properties, non toxicity and less price (Vij *et al*., 2001; Mondal *et al*., 2002). First time in history, Murashige (1978) used calcium alginate matrix as coating material for somatic embryos.

The use of diverse substances (agar, gel rite & DMSO) to encapsulate somatic embryos, with sodium alginate and calcium chloride solution lead to the most excellent outcomes for synthetic seeds formation (Maruyama *et al*., 2003; Utomo *et al*., 2008). Malek *et al.,* (2009) encapsulated shoot tips of pointed gourd in sodium alginate matrix. Asmah *et al*., (2011) reported different material like hydro gel, alginate gel, dimethyl sulfoxide (DMSO) and ethylene glycol, for encapsulation of shoot tips, axillary buds and somatic embryos to develop synthetic seeds. Various concentrations of sodium alginate having dimensions from 1.5% to 6% have been used for various researchers such as 2% to 5% Na-alginate (w/v) (Murashige & Skoog, 1962; Asmah *et al*., 2011), 2% Na-alginate (Rady & Hanafy, 2004; Reddy *et al*, 2005; Latif *et al*., 2007; Cartes *et al*., 2009), 2.5% Na-alginate (Geetha & Gopal, 2009), 3% Na-alginate (Cartes *et al*., 2009; Tabassum *et al*., 2010; Sarmah *et al*., 2010), and 4% Na-alginate (Rizkalla *et al*., 2012). Similarly various concentrations of calcium chloride as complexing agent had been reported by several researchers like 25mM to 100mM $CaCl_2.2H_2O$ solutions (Murashige & Skoog, 1962; Asmah *et al*., 2011) 25µm to 100µm (Priya & Shakila, 2003); 75mM (Latif *et al*., 2007); 150µM $CaCl_2$ solution (Reddy *et al*., 2005), 100mM (Tabassum *et al*., 2010; Sarmah *et al*., 2010) and 50mM to 200mM (Ali *et al*., 2013) respectively.

Synthetic seed technology is a good replacement to conventional agricultural practices applied to enhance plant propagation and in different non seed producing plants and crops. It is also more beneficial because of its less price and easy access to obtain germplasm, ultimately help in establishing plant nurseries on large scale. There is

also a great possibility of manufactured seeds in important economic crops in which viable seed formation is difficult. Furthermore, synthetic seeds production is useful not only for germplasm construction, but also to study the liberality of man-made seeds to abiotic stresses, in particular, soil salinity, drought, water logging and heat stress which are generally harmful to plant growth and yield in Pakistan and worldwide. A number of studies have been accomplished on synthetic seeds development in various plants but still there is a lack of information on effect of sodium alginate and calcium chloride treatments on nature, colour, and quality of encapsulation and survival rate of manufactured seeds. Thus, in an attempt to enhance synthetic seeds production, the current study was conducted to assess the effects of sodium alginate as encapsulating agent and calcium chloride as complexing agent treatments on type, colour, quality of encapsulation and survival rate of synthetic seeds.

Materials and Methods

Experimental site: *In vitro* study was conducted to evaluate the efficient synthetic seeds formation of sugarcane (*Saccaharum officinarum* var. NSG-59) from somatic embryos by using sodium alginate (Na-alginate) and calcium chloride ($CaCl_2$) in PTC Lab, Department of Botany, University of Sargodha, Sargodha, Pakistan.

Plant material: Somatic embryos of sugarcane (*Saccaharum officinarum* var. NSG-59) were taken as source of plant material for production of synthetic seeds. Somatic embryos were produced *In vitro* by following protocol described by Iqbal *et al.,* (2016).

Preparation of encapsulation matrix and complexing agent: Various levels of Na-alginate (0.5%, 1%, 2%, 2.5%, 3%, 3.5%, and 4%) as gel matrix were synthesized for encapsulation. To make homogenous mixture, the gel matrix was heated on hot plate with continuous stirring. A complexing agent ($CaCl_2$ solution) was prepared in range of 50mM, 70mM, 90mM, 100mM, 150mM and 200mM in distilled water. Both gelling (Na-alginate) and complexing agents ($CaCl_2$) were sterilized in autoclave before use.

Isolation of somatic embryos: Somatic embryos were transferred to MS (Murashige & Skoog, 1962) liquid medium in jars inside laminar air flow cabinet through sterile forcep. For the preparation of MS medium, exact quantities of all the components (Macronutrients, Micronutrients, Iron EDTA and Vitamins) were mixed properly. About 30g sucrose was added and final volume of the medium (1L) was made by the addition of double distilled water. The pH of the medium was adjusted between 5.7-5.8 by adding drops of 1N HCl and 1N NaOH. The jars were positioned on orbital shaker at 60 rpm for 6 hours to isolate somatic embryos from each other. The entire process was done at room temperature under sterile conditions.

Encapsulation of somatic embryos: Encapsulation of somatic embryos was done inside the laminar air flow cabinet by using Na-alginate ($NaC_6H_7O_6$) as gelling matrix and $CaCl_2$ as complexing agent. Dropping method was used for encapsulation in which sodium alginate drop was applied on single somatic embryo and then it allowed falling in $CaCl_2$ solution. The dropped embryo (containing gel matrix) was kept in $CaCl_2$ solution for 5 to 25 minutes for successful encapsulation. At the end the beads were collected and washed thrice with distilled water to eliminate remains of $CaCl_2$ solution. The produced synthetic seeds were further stored at 4^{o}C in refrigerator.

Results and Discussions

Role of sodium alginate (Na-alginate) and calcium chloride ($CaCl_2$) on type, colour, quality of encapsulation and survival rate of encapsulated somatic embryos: Various levels of sodium alginate (Na-alginate) ranging from 0.5% to 4% and calcium chloride ($CaCl_2$) solution of different concentration i.e. 50mM, 70mM, 90mM, 100mM, 150mM and 200mM were used in present experiment (Table 1; Fig. 1). The data presented in Table 1 showed that 0.5% Na-alginate failed to show encapsulation with all concentrations of $CaCl_2$ solution. The increase in concentration of Na-alginate up to 1% with 150mM $CaCl_2$ solution resulted in poor beads formation and decreased embryo survival rate (60%). However firm, apparent and isodiametric beads were formed in 3% Na-alginate + 100mM $CaCl_2$ solution with 95% embryo survival rate (Table 1; Fig. 1). The data (Table 1) depicted loose and transparent encapsulation with 2% Na-alginate concentration + 100mM $CaCl_2$ solution and resulted transparent, soft and fragile beads with 75% embryo survival rate. However by further increasing in concentration of both Na-alginate and $CaCl_2$ solution, gradual improvement in results was noticed. The embryo survival rate was 85% in response to 2.5% Na-alginate + 150mM $CaCl_2$ solution. Almost similar results were obtained when 200mM $CaCl_2$ solution was used. The data presented in Table 1 showed that best result was obtained in 3% Na-alginate and 100mM $CaCl_2$ solution where compact isodiametric and transparent beads were obtained with 95% embryo survival rate. Further increase in concentration of $CaCl_2$ solution with constant Na-alginate value produced very rigid and firm beads. As a result, the regeneration of somatic embryos was decreased to 70%. Any further increase in concentration of Na-alginate did not prove good results for synthetic seeds formation (Table 1). In present study synthetic seeds were obtained by encapsulating somatic embryos with Na-alginate used as gelling agent and $CaCl_2$ solutionas complexing agent (Table 1; Fig. 1). Similar results have been reported in previous studies (Singh *et al*., 2006; Latif *et al.*, 2007; Malek, 2009; Cartes *et al*., 2009; Tabassum *et al*., 2010; Asmah *et al*., 2011; Ali *et al*., 2012). Different types of encapsulating agents can be used for encapsulation of somatic embryos. Asmah

et al., (2011) reported different material like hydro gel, alginate gel, ethylene glycol, dimethyl sulfoxide (DMSO) for encapsulation of somatic embryos. In present investigation, Na-alginate was used due to its easy gelling properties and non toxic nature. Vij *et al*., (2001) and Mondal *et al*., (2002) also preferred Na-alginate gel matrix because of its uncomplicated gelling capacity, non toxicity and low cost. It was observed in present work that 3%, 3.5% and 4% Na-alginate in 100mM, 90mM and 90mM $CaCl_2$ solution respectively were found to be optimum for formation of isodiametric and compact beads with maximum rate of survival (95%) and excellent encapsulation quality (Table 1; Fig. 1). Cartes *et al*., (2009) used 2%, 3%, and 4% Na-alginate as encapsulation material and $CaCl_2$ solution as complexing agent. Similarly Ali *et al*., (2012) reported gel matrix of Na-alginate in the range of 1%, 2%, 3%, 4% and 5% and $CaCl_2$ solution as complexing agent, in the range of 50mM, 100mM, 150mM and 200mM for encapsulation. Sarmah *et al*., (2010) tested different concentrations of Na-alginate and reported that 3% Na-alginate treated with 100mM $CaCl_2.2H_2O$ solution for 30 minutes produced firm, clear, round and uniform optimal beads. Similarly Ali *et al*., (2012) reported 3% Na-alginate along with 100mM $CaCl_2$ solution as more feasible for the formation of firm, clear and isodiametric ideal beads.

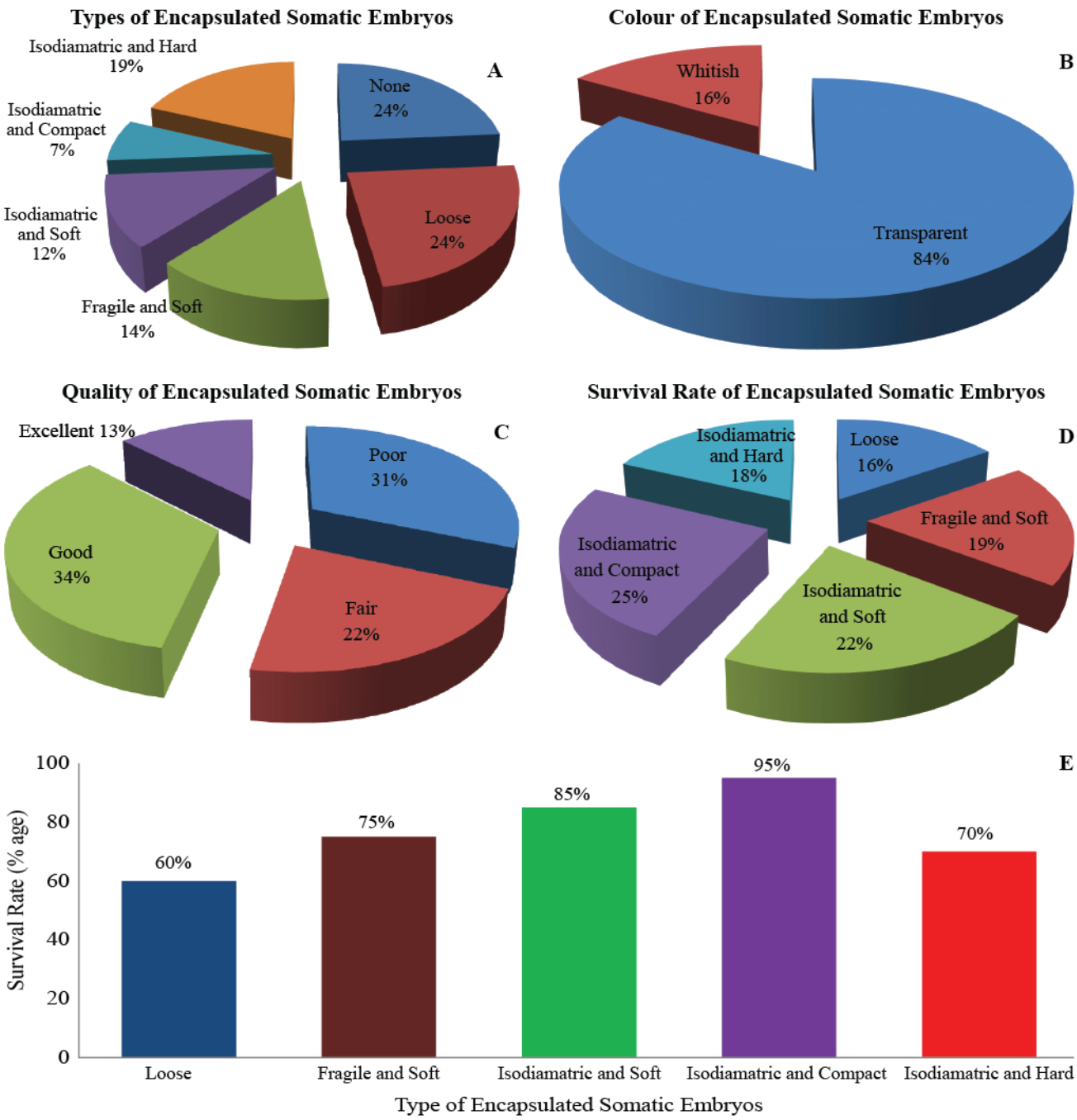

Fig. 1. Effect of Na-alginate and $CaCl_2$ on (A) Type of encapsulated somatic embryos (B) Colour of encapsulated somatic embryos (C) Quality of encapsulated somatic embryos (D, E) Survival rate of encapsulated somatic embryos.

Table 1. Effect of Na-alginate and calcium chloride ($CaCl_2$) on type, colour, quality of encapsulation and survival rate of encapsulated somatic embryos.

Sr. No.	Media Composition Na-Alginate+$CaCl_2$ (mM)	Type of Encapsulation	Color of Encapsulation	Encapsulation Quality	Survival Rate (%age)
1.	0.5 + 50	No	----	----	----
2.	0.5 + 70	No	----	----	----
3.	0.5 + 90	No	----	----	----
4.	0.5 + 100	No	----	----	----
5.	0.5 + 150	No	----	----	----
6.	0.5 + 200	No	----	----	----
7.	1 + 50	No	----	----	----
8.	1 + 70	No	----	----	----
9.	1 + 90	No	----	----	----
10.	1 + 100	No	----	----	----
11.	1 + 150	Loose	Transparent	+	60
12.	1 + 200	Loose	Transparent	+	60
13.	2 + 50	Loose	Transparent	+	60
14.	2+ 70	Loose	Transparent	+	60
15.	2+ 90	Loose	Transparent	+	60
16.	2 + 100	Fragile and soft	Transparent	++	75
17.	2+ 150	Fragile and soft	Transparent	++	75
18.	2 + 200	Isodiametric and soft	Transparent	+++	85
19.	2.5 + 50	Loose	Transparent	+	60
20.	2.5 + 70	Loose	Transparent	+	60
21.	2.5 + 90	Fragile and soft	Transparent	++	75
22.	2.5 + 100	Fragile and soft	Transparent	++	75
23.	2.5 + 150	Isodiametric and soft	Transparent	+++	85
24.	2.5 + 200	Isodiametric and soft	Transparent	+++	85
25.	3 + 50	Loose	Transparent	+	60
26.	3 + 70	Fragile and soft	Transparent	++	75
27.	3 + 90	Isodiametric and soft	Transparent	+++	85
28.	3 + 100	Isodiametric and compact	Transparent	++++	95
29.	3 + 150	Isodiametric and hard	Transparent	+++	70
30.	3 + 200	Isodiametric and hard	Whitish	+++	70
31.	3.5+ 50	Loose	Transparent	+	60
32.	3.5 + 70	Fragile and soft	Transparent	++	75
33.	3.5 + 90	Isodiametric and compact	Transparent	++++	95
34.	3.5 + 100	Isodiametric and hard	Transparent	+++	70
35.	3.5 + 150	Isodiametric and hard	Transparent	+++	70
36.	3.5 + 200	Isodiametric and hard	Whitish	+++	70
37.	4 + 50	Loose	Transparent	+	60
38.	4 + 70	Isodiametric and soft	Transparent	++	85
39.	4 + 90	Isodiametric and compact	Transparent	++++	95
40.	4 + 100	Isodiametric and hard	Whitish	++++	95
41.	4 + 150	Isodiametric and hard	Whitish	+++	70
42.	4 + 200	Isodiametric and hard	Whitish	+++	70

Poor = +; Fair = + +; Good = + + +; Excellent = + + + +

Table 2. Effect of treatment time of $CaCl_2$ for synthetic seeds formation.

Medium	Treatment time (min)	Type of encapsulation	Quality of encapsulation
2% Na-Alginate + 100mM $CaCl_2$	5	Fragile soft	+
	10	Fragile soft	+
	15	Isodiametric soft	+ +
	20	Isodiametric soft	+ +
	25	Isodiametric soft	+ +
3% Na-Alginate + 100mM $CaCl_2$	5	Fragile soft	+
	10	Isodiametric soft	+ +
	15	Isodiametric compact	+ + + +
	20	Isodiametric hard	+ + +
	25	Isodiametric hard	+ + +
4% Na-Alginate + 100mM $CaCl_2$	5	Isodiametric soft	+ +
	10	Isodiametric compact	+ + + +
	15	Isodiametric hard	+ + +
	20	Isodiametric hard	+ + +
	25	Isodiametric hard	+ + +

Poor = +; Fair = + +; Good = + + +; Excellent = + + + +

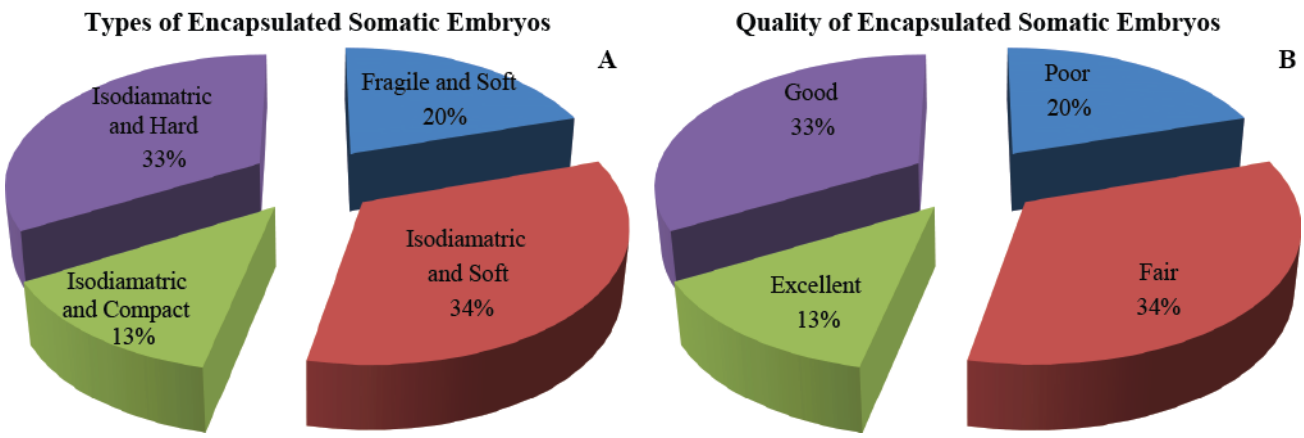

Fig. 2. Effect of treatment time of $CaCl_2$ on (A) Types of encapsulated somatic embryos (B) Quality of encapsulated somatic embryos.

Role of $CaCl_2$ application time interval on encapsulation: In order to check the type of encapsulation and beads quality somatic embryos were subjected to different concentration of Na-alginate at different time intervals. It was proved that beads quality was independent of the duration of Na-alginate treatment, where as treatment time of $CaCl_2$ solution affected the beads quality (Table 2; Fig. 2). Better quality seeds (isodiametric and compact) were produced at 3% Na-alginate as compared to others (0.5%, 1%, 2%, 2.5%, 3%, 3.5% and 4%) when treated with 100mM $CaCl_2$ solution for the period of 15 minutes. It was noticed in present study that the decrease in the treatment time of $CaCl_2$ solution (≤ 10 min) resulted in delicate and malleable beads formation. Synthetic seeds became hard white color when treated $CaCl_2$ solution for 20 minutes. This hardness caused failure in somatic embryos germination by rendering them dried. It was found in present study that duration of Na-alginate had no effect but treatment time of $CaCl_2$ solution influenced beads formation (Table 1; Fig. 2). According to Ali *et al*., (2012) Na-alginate treatment time has no significant effect on beads formation, however, $CaCl_2$ solution treatment proved to be crucial. Present work also highlighted that 3% Na-alginate and 100mM solution of $CaCl_2$ solution proved best for isodiametric beads formation (Table 1; Fig. 2). Tabassum *et al*., (2010) reported encapsulation mixture containing 3% Na-alginate along with 100mM $CaCl_2$ solution and 1/4 volume of the cell suspension nutrient mixture containing 5×10^{-4} somatic embryos per ml proved to be the best for encapsulation. Sarmah *et al*., (2010) suggested that among different concentrations of Na-alginate, 3% concentration with exposure to 100mM $CaCl_2$ solution for 30 min produced firm, clear, round and uniform optimal beads. In present study maximum i.e., 95% survival rate of embryos was recorded at 3%, 3.5% and 4% Na-alginate in 90mM and 100mM $CaCl_2$ (Table 1). Bekheet (2006) reported that 3% sodium alginate as gel matrix showed highest survival rate of embryos and conversion of encapsulated bulblets to plantlets. He further demonstrated that 30 minutes was the best exposure time for hardening of gel beads.

Conclusion

The protocol has successfully been established for rapid conversion of somatic embryos by using Na-alginate and $CaCl_2$ for the production of synthetic seeds of sugarcane (*Saccharum officinarum* L.). To enhance the synthetic seeds production, methods for synchronization of developing propagules should be consistent throughout encapsulation process. The synthetic seeds provide feasible handling and transport of seedlings or plants and can also be used for large scale production of viable plants of sugarcane by direct sowing of synthetic seeds in the soil. The protocol established in present investigation can further be used for studies of synthetic seeds with respect to their viability and storage quality for a long period of time. However, commercial exploitation of the protocol is yet to be addressed for other crops and plant species that failed to produce viable seeds or difficult to propagate through conventional means.

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