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GROWTH RESPONSE OF FERTILIZED HYACINTH BEAN (*DOLICHOS LABLAB* L.) OVULES TO DIFFERENT CULTURE MEDIA

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SUMMARY

Fertilized ovules of hyacinth bean (*Dolichos lablab* L.) were cultured after 6, 8, 10 and 12 days of anthesis in ten different culture media devised earlier for the culture of pollinated ovaries or ovules or developing embryos in Murashige and Skoog (1962) medium with modifications. Growth response, measured after 10 days in culture, as increase in length, breadth, fresh and dry weight, concentration of sugars, starch and protein was maximum in Monnier's (1978) medium in ovules cultured at 8 days after anthesis. Ovules cultured at 6 days after anthesis aborted within 3-4 days of culture.

***Key words*: Culture media, fertilized ovules, hyacinth bean, starch, sugars.**

INTRODUCTION

Ovule culture is a promising tool for raising hybrid seeds, which normally fail to develop due to embryo abortion at early stages of development. It can also be useful for investigation of the role of nutrients in embryo and seed development. Several modifications of the culture media have been practiced for developing seeds from excised ovules containing zygotic proembryo with a few endosperm nuclei. These modification are high osmolarity (4-10% sucrose) of the culture medium (Welbaum and Bradford 1988), amino acids as nitrogen source (Kapoor 1959), GA_3 and natural extracts such as coconut milk (Kapoor 1959), young fruit juice of cucumber or watermelon (Wakizuka and Nakajima 1974) casein hydrolysate or casamino acids (Pontovich and Sveshnikova 1966) to previously defined culture media.

The effect of placental tissue is also beneficial for the normal growth of ovules in culture (Rangaswamy and Shivanna 1971). Steward and Hsu (1978) devised a medium for the culture of cotton ovules for raising hybrid embryo of interspecific crosses. Monnier's (1978) formulation contained increased levels of K, Ca and sucrose and reduced level of NH_4^+ ions for the culture of excised *Capsella* embryo. The B_5 medium (Gamborg *et al.* 1968) was described suitable for the culture of soybean embryo. The addition of the plant hormone ABA has been suggested later to continue the developmental processes and prevent precocious germination of embryos (Kermode 1990).

This paper reports standardization of the technique for the culture of hyacinth bean ovules excised 6, 8, 10 and 12 days after anthesis. Several modifications of some predefined tissue culture media, without the growth factors were examined for continued growth of the ovules. The suitability of the media was judged on the basis of increase in their dimensions, fresh and dry weight and change in the level of sugars, starch and proteins in the developing seeds.

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MATERIALS AND METHODS

Plants of hyacinth bean (*Dolichos lablab* L.) were grown in field under natural conditions. The developing pods were harvested at different intervals for judging the appropriate stage and the most suitable medium for culture of embryo in intact ovule up to seed formation. The developing pods were harvested at 6, 8, 10 and 12 days after anthesis (DAA) and developing seeds (fertilized ovules) were used as explant.

All glassware and culture media were sterilized by autoclaving at 103 KPa pressure for 30-45 minutes. For surface sterilization, the explant was washed at first with diluted triton-X followed by running tap water and treated for five minutes with 0.1% mercuric chloride. Mercuric chloride was removed from the surface by repeated washings (4-5 times) with sterile double distilled water. Pods were cut from the sides with the help of an aseptic scalpel and forceps, carefully opened and ovules along with placenta were excised from pod and inoculated carefully. All the processes as sterilization of explant, transfer of media to petriplates, excision and transfer of explants, were carried out under a laminar airflow cabinet provided with HEPA filters and UV lamp.

The composition of culture media used in the study was as follows:

M_1- MS (Murashige and Skoog 1962) medium with casein hydrolysate (250 mg l^{-1}).

M_2- MS medium with asparagine (400 mg l^{-1}).

M_3- MS medium with amino acid mixture of Norstog (1973).

M_4- MS medium with casein hydrolysate, asparagine and amino acid mixture at the above mentioned concentrations.

M_5 - Kanta and Maheshwari's (1963) medium.

M_6- Monnier's (1978) medium.

M_7- Monnier's (1978) medium with only 5% sucrose.

M_8- Nitsch's (1969) basal medium with vitamin mixture of White (1953).

M_9- Steward and Hsu's (1978) medium with 0.7% agar.

M_{10}- B5 (Gamborg *et al.* 1968) medium.

No growth regulators were supplied in the above-mentioned media. The pH of media was adjusted to 5.6 before autoclaving. The culture containers after transfer of the explant were sealed with parafilm and kept in a BOD incubator provided with lights of 70Wm^{-2} for 16h photoperiods.

Growth responses as length, breadth, fresh and dry weight of developing ovules was observed before and after 10 days of culture.

The concentration of sugars and starch was determined in ovules or seeds before and after 10 days of culture. Samples were fixed in 80% (v/v) boiling ethanol and ground at room temperature with a pestle and mortar. The alcohol soluble and insoluble fractions were separated by centrifugation at 2000xg for 15 minutes. Sugars from the alcohol soluble fraction were transferred to aqueous phase and de-pigmented by using lead acetate. Excess lead from the solution was precipitated with di-sodium hydrogen phosphate. Reducing and total sugars in this solution were determined (Nelson 1944), before and after hydrolysis with 1% (w/v) invertase at 4°C for 12 hrs. The concentration of non-reducing sugars was calculated by subtracting the value of reducing sugars from total sugars. Starch from the alcohol insoluble fraction was extracted by maceration in perchloric acid and content was determined colorimetrically by using phenol and hot concentrated sulphuric acid according to the method of Montgomery (1957). The data were analyzed statistically for the standard error (S.E.).

Total proteins were determined in ovules before and after 45 days in culture in the depigmented powdered material. Proteins were extracted in 50mM Tris-HCl buffer pH 6.8 containing 2% SDS, 5% (w/v) ß-mercaptoethanol, 10% (v/v) glycerol and 10mM $MgCl_2$. The extract was centrifuged at 15,000xg at 4°C for 20 minutes and proteins content of the supernatant was determined by the method of Lowry *et al.* (1951). The proteins in solution were denatured by heating in a boiling

water bath for 3 minutes and fractionated in discontinuous 12% SDS-PAGE in a mini gel electrophoresis apparatus according to the method of Laemmli (1970).

RESULTS AND DISCUSSION

Fertilized ovules of hyacinth bean, cultured at 8, 10 and 12 DAA (days after anthesis), showed growth stimulation, while those at 6 DAA aborted within 3-4 days after culture. Out of the ten culture media, distinct growth enhancement in ovules of hyacinth bean was observed in M_3, M_5, M_6 and M_7 media (Table1). The media M_6 (Monnier 1978) appeared best for the growth of hyacinth bean ovules whereas, M_5 (Kanta and Maheshwari 1963), M_7 (Monnier's medium with only 5% sucrose) and M_3 (MS without growth regulators and with amino acid mixture of Norstog 1953) also showed good growth of ovules in culture.

The most pronounced growth enhancement was observed in ovules cultured at 8 DAA as compared to their initial weight before culture (Table 2). The culture of fertilized hyacinth bean ovules resulted in the increased level of both reducing and non-reducing sugars in ovules at 8 DAA in M_6, M_8, M_5 and M_7 media (Table 3). In ovules cultured at 10 and 12 DAA in media other than M_6, the total and non-reducing sugars decreased from

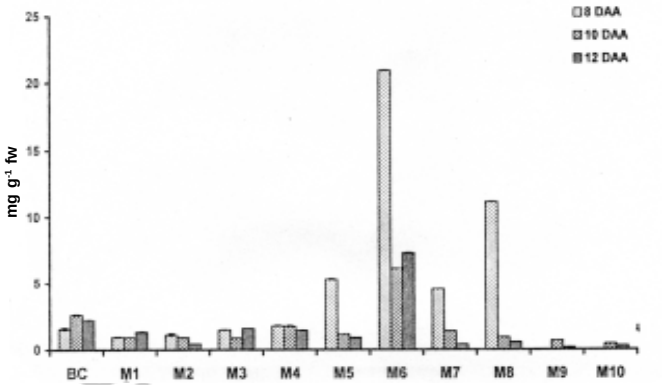

Fig. 1. Concentration of total sugars (±S.E., n=3) in hyacinth bean ovules before and after 10 days of culture in different media.

Table 1. Length and breadth (± S.E., n = 5) of hyacinth bean ovules before and after 10 days of culture in different media.

Medium	Days after anthesis					
	8	10	12	8	10	12
	Length (mm)			Breadth (mm)		
Before culture						
	4.0 ± 0.11	6.0 ± 0.10	9.0 ± 0.03	1.9 ± 0.00	2.7 ± 0.02	4.2 ± 0.06
After culture						
M_1	4.0 ± 0.06	7.0 ± 0.12	9.5 ± 0.02	3.0 ± 0.03	3.5 ± 0.03	5.0 ± 0.06
M_2	6.0 ± 0.06	5.0 ± 0.06	9.0 ± 0.03	2.0 ± 0.14	2.0 ± 0.14	4.0 ± 0.05
M_3	6.0 ± 0.07	6.0 ± 0.07	10.0 ± 0.07	2.0 ± 0.10	2.0 ± 0.14	5.0 ± 0.07
M_4	5.0 ± 0.06	8.4 ± 0.02	9.3 ± 0.02	2.0 ± 0.14	2.5 ± 0.05	5.0 ± 0.05
M_5	6.8 ± 0.03	6.0 ± 0.07	9.8 ± 0.03	3.2 ± 0.03	4.0 ± 0.03	5.4 ± 0.07
M_6	8.1 ± 0.05	11.8 ± 0.03	13.0 ± 0.03	3.5 ± 0.03	5.0 ± 0.05	6.0 ± 0.05
M_7	7.8 ± 0.01	8.0 ± 0.07	12.2 ± 0.03	3.2 ± 0.06	4.5 ± 0.05	4.5 ± 0.05
M_8	5.5 ± 0.07	6.5 ± 0.03	10.0 ± 0.11	2.0 ± 0.06	2.8 ± 0.03	4.0 ± 0.06
M_9	4.8 ± 0.06	6.9 ± 0.00	10.0 ± 0.07	1.9 ± 0.02	3.0 ± 0.03	5.0 ± 0.07
M_{10}	5.0 ± 0.06	7.0 ± 0.3	10.1 ± 0.03	2.2 ± 0.06	2.7 ± 0.03	4.5 ± 0.05

Table 2. Fresh and dry weight (± S.E., n = 3) of hyacinth bean ovules before and after 10 days of culture in different media.

Medium	Days after anthesis					
	8	10	12	8	10	12
	Fresh wt. (mg ovule^{-1})			Dry wt. (mg ovule^{-1})		
Before culture						
	10 ± 0.0	34 ± 0.5	85 ± 0.0	1.0 ± 0.1	3.2 ± 0.0	8.0 ± 0.1
After culture						
M_1	10 ± 0.0	27 ± 0.2	75 ± 0.5	1.0 ± 0.1	3.0 ± 0.1	8.3 ± 0.1
M_2	14 ± 0.5	25 ± 0.5	83 ± 0.6	1.5 ± 0.2	2.8 ± 0.1	9.2 ± 0.5
M_3	14 ± 2.0	39 ± 0.5	90 ± 0.2	1.5 ± 0.1	4.3 ± 0.0	10.0 ± 0.2
M_4	12 ± 1.6	32 ± 0.2	80 ± 0.5	1.3 ± 0.5	3.5 ± 0.5	8.9 ± 0.2
M_5	14 ± 1.3	45 ± 0.6	90 ± 0.6	1.6 ± 0.1	5.0 ± 0.5	9.9 ± 0.1
M_6	21 ± 0.5	44 ± 0.2	97 ± 0.6	2.3 ± 0.1	4.8 ± 0.2	10.0 ± 0.1
M_7	12 ± 0.1	36 ± 0.6	91 ± 0.5	1.3 ± 0.2	4.0 ± 0.2	10.0 ± 0.2
M_8	12 ± 0.6	24 ± 0.1	83 ± 0.6	1.3 ± 0.2	2.7 ± 0.2	9.2 ± 0.5
M_9	11 ± 0.6	18 ± 0.2	80 ± 0.2	1.2 ± 0.1	2.0 ± 0.1	8.9 ± 0.5
M_{10}	12 ± 0.2	30 ± 0.5	80 ± 0.2	1.3 ± 0.2	2.0 ± 0.1	9.0 ± 0.2

their previous values observed before culture (Table 3 & Fig. 1). The increase in both reducing and non-reducing sugars in ovules cultured at 8 DAA might be due to increase in uptake and utilization of sugars in ovules cultured for the synthesis of different seed reserves including the RFO's (Raffinose family of oligosaccharides).

The RFO's are known to protect cellular structures during desiccation and constitute carbon reserves for germination. RFO's accumulation in seeds is controlled by its initial substrates myo-inositol and sucrose (Karner *et al*. 2004). Sugars have been suggested to be modulators of source and sink interaction in maize (Foyer 1988). In tissue culture grain setting has been shown to be carbohydrate dependent (Cobb *et al.* 1988, Lee *et al.* 1989). The enhancement of sugars was the most in M_6 medium that contained 12% sucrose, greater than the other media used in the study. The results support the view that high osmolarity enhances *in vitro* growth of embryo partially substituting the ABA requirement and inducing the desiccation tolerance (Xu *et al*. 1990). High osmolarity of culture medium has been shown important for development of excised ovules of *Petunia hybrida* (Wakizuka and Nakajima 1974).

The concentration of starch also increased in cultured ovules from that observed before culture (Fig. 2). The enhancement in the starch content was greater in ovules cultured at 12 days after anthesis in all media except in M_7, M_8, M_9 and M_{10}. The increase in starch content in cultured seeds was most pronounced in M_5 and M_6 media and further substantiates their suitability for the culture of hyacinth bean ovules. The growth enhancement of ovules with increased starch content in the Monnier's (1978) medium (M_6) might be due to higher (12%) sucrose supply stimulatory for the synthesis of starch by regulating ADPG pyrophosphorylase and starch synthetase (Hannah 1997).

Table 3. Concentration of reducing and non-reducing sugars (± S.E., n = 3) in hyacinth bean ovules before and after 10 days of culture in different media.

Medium	Days after anthesis					
	8	10	12	8	10	12
	Reducing sugars (mg g^{-1} fw)			Non-Reducing sugars (mg g^{-1} fw)		
Before culture						
	0.86 ± 0.02	0.44 ± 0.02	0.42 ± 0.02	0.72 ± 0.01	2.52 ± 0.10	1.80 ± 0.06
After culture						
M_1	0.40 ± 0.01	0.49 ± 0.00	0.63 ± 0.02	0.54 ± 0.01	0.43 ± 0.02	0.70 ± 0.09
M_2	0.27 ± 0.01	0.33 ± 0.03	0.22 ± 0.04	0.84 ± 0.01	0.58 ± 0.01	0.22 ± 0.01
M_3	0.63 ± 0.02	0.32 ± 0.02	0.50 ± 0.01	0.85 ± 0.01	0.84 ± 0.01	0.97 ± 0.01
M_4	0.99 ± 0.00	0.92 ± 0.02	0.58 ± 0.01	0.78 ± 0.01	0.85 ± 0.23	0.87 ± 0.01
M_5	3.15 ± 0.11	0.53 ± 0.09	0.54 ± 0.02	2.14 ± 0.13	0.62 ± 0.09	0.35 ± 0.01
M_6	7.08 ± 0.80	3.05 ± 0.21	2.82 ± 0.14	13.87 ± 0.12	3.09 ± 0.06	4.52 ± 2.07
M_7	1.17 ± 0.08	0.47 ± 0.01	0.16 ± 0.02	3.37 ± 0.09	0.92 ± 0.01	0.12 ± 0.01
M_8	5.48 ± 0.19	0.23 ± 0.02	0.12 ± 0.08	5.66 ± 0.09	0.70 ± 0.01	0.45 ± 0.01
M_9	-	0.51 ± 0.03	0.03 ± 0.02	-	0.16 ± 0.01	0.13 ± 0.01
M_{10}	-	0.14 ± 0.01	0.12 ± 0.02	-	0.29 ± 0.01	0.17 ± 0.00

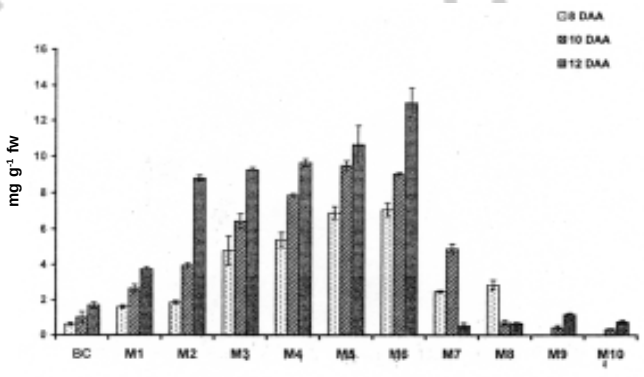

Fig. 2. Concentration of starch (±S.E., n=3) in hyacinth bean ovules before and after 10 days of culture in different media.

Good growth response by cultured ovules in the Monnier media (M_6 and M_7) could also be due to higher concentration of Ca^{++} and K^+ and glutamine as nitrogen source along with higher sucrose concentration (>5%). The suitability of Kanta and Maheshwari's (1963) medium might be due to Fe citrate as Fe source or glycine at higher and inorganic salts at lower concentration.

Total proteins in cultured ovules increased from the values observed before culture at 8, 10 and 12 DAA (Fig. 3). The increase in protein content was more in ovules cultured on 8 DAA (Fig. 3). The SDS-PAGE profile of

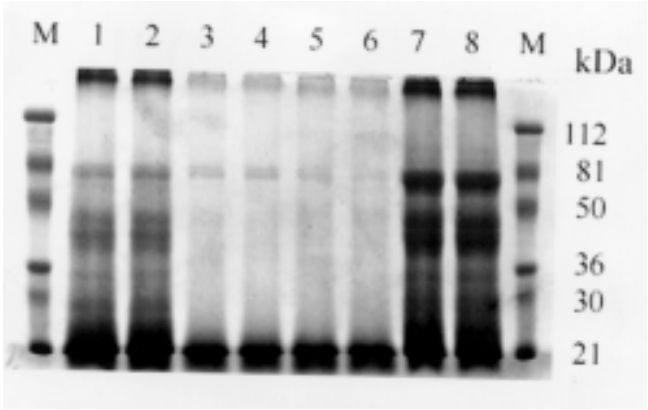

Fig. 3. SDS-PAGE protein profile of hyacinth bean ovules cultured in Monniers's medium at 8 DAA (Lane 1,2), 10 DAA (Lane 3,4), 12 DAA (Lane 5,6) as compared to matured seed (Lane 7,8). Lane M (left and right), protein molecular weight markers in kDa.

total proteins indicates the accumulation of almost all seed storage proteins in ovules cultured at 8 DAA and not in those cultured at 10 and 12 DAA. The protein profile of ovules cultured at 8 DAA resembled that of seeds developed in *planta* under natural conditions but their concentration was comparatively less than seed proteins synthesized in natural conditions. In M_6 medium almost all seed storage proteins were synthesized only in the ovules cultured at 8 DAA. This indicated that in ovules at 10 and 12 DAA utilization of sugars for the synthesis of starch and proteins was disturbed and proteins other than storage proteins were synthesized (Marschner 1995).

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