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MATURE ZYGOTIC EMBRYO CULTURE OF *CAPSCICUM ANNUUM* L. A COMMERCIALLY IMPORTANT PLANT

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Abstract:

Zygotic Embryo culture is the aseptic isolation and growth of sexually embryos in vitro with the objective of obtaining viable plants. The aim of this study was to evaluate the *in vitro* germination of zygotic embryos from mature seeds of Green Pepper *Capsicum annuum*.L cv CA960 and the morphogenetic responses of the explant to different concentrations of growth regulators. Excision of the zygotic embryos was inoculated in Murashige and Skoog medium, supplemented with 6-benzylaminopurine (3.0 mg/L). Important effect of 6-benzylaminopurine in adventitious bud formation was demonstrated. All Inoculated test tubes were kept in a growth room at a temperature of 25 ± 2 ºC for 16 hours of photoperiod for 30 days. The Zygotic embryo culture had a higher *in vitro* germination rate than the normal seed germination in nature. However, zygotic embryos in MS medium supplemented with (2.0 mg/L) BAP had the highest percentage of regeneration (80%), number of shoots (3.25), buds (2.85) and leaves (3.15), multiplication rate (27.75), and length of shoots (1.96 cm). The *in vitro* culture of zygotic embryos made possible to the multiplication of a higher number of healthy *in vitro* seedlings. Thus, it can be used as an alternative technique for the propagation of this species.

Keywords: Green Pepper, *Capsicum annuum* L; cv CA960, Zygotic Embryos. *in vitro* culture, bud induction, plant conformity morphogenesis;

Introduction:

Pepper (*Capsicum* sp.) is one of the most varied and widely used foods in the world. From the various colors to the various tastes, peppers are an important spice commodity and an integral part of many cuisines. Peppers originated in the Mexico and Central America regions. Christopher Columbus encountered pepper in 1493 and, because of its pungent fruit, thought it was related to black pepper, Piper *nigrum*, which is actually a different genus. Nevertheless, the name stuck and he introduced the crop to Europe, and it was subsequently

spread into Africa and Asia. Peppers were important to the earliest inhabitants of the western hemisphere as much as 10,000 to 12,000 years ago. Plant remnants have been found in caves in the region of origin that date back to 7,000 B.C. The Incas, Aztec and Mayans all used pepper extensively and held the plant in high regard. Many of the early uses of pepper centered on medicinal purposes. Pepper has been credited with any number of useful cures and treatments, some of which are valid and some of which are probably more folklore.

Peppers are the fruits of plants belonging to the genus *Capsicum* of the Nightshade family (Solanaceae). The genus consists of about 25 wild and 5 domesticated species (Sanatombi and Sharma, 2007). However, *Capsicum annuum* L. is considered as the economically most important species of the genus. The species includes both mild and pungent fruit types. In conventional systems seeds are generally utilized for multiplication and production. This method has some disadvantages such as: short viability period, low rate of germination, high risk of catching various diseases. In addition, pepper is sensitive to many pathogens and pests, including fungi, bacteria, viruses and nematodes and to extreme climatic conditions, especially temperature extremes, that are limiting factors for its production (Christopher and Rajam, 1994; Agrawal *et al*., 1988). In order to improve propagation of the commercial cultivars of these species and to meet the increasing demands for the crops, more reliable propagation approaches for mass multiplication are needed. Tissue culture methods provide a way to asexually multiply pepper plants as the plants lack natural vegetative propagation.

Materials and methods

Plant material: *Capsicum annuum*.L cv CA960 was used in our study. It was selected by the Agriculture Research Institute Warangal. This variety is sensitive to viruses mainly PVMV, CMV and PVY (Khadmaoui, 1996). var- Pusa ruby is characterized by a semi-long conic fruit with a narrow base and obtuse end. Fruit has a pungent taste and becomes red when ripe.

Explants: We used mature zygotic embryos, excised from seeds.

Excision of embryo: For the *in vitro* culture of mature zygotic embryos generally it is necessary to free them from their surrounding tissues. Mature seeds of pepper were soaked in water for 24 hours and surface sterilized using commercial sodium hypochlorite (8%) for 10 minutes. They were then washed three times in sterile distilled water. Before culture, the external seed envelope was removed under a microscope and the embryo was excised by a slight pressure at the cotyledon level. Intact embryos were transferred directly to a sterile test tube congaing plate containing 6-benzylaminopurine (3.0 mg/L).

Important aspect for technique: Two most important aspect of zygotic embryo is

1. Composition of the culture medium
2. Excision of the embryo

Technique: The choice of plant material may become important when the objective is to introduce the technique to beginner

Plant Material:

- Embryo which can be easily dissected out should select.
- Mature embryo of seed Green pepper
- At the same stage of development large number of genetically uniform embryos can be obtained.
- Surface Disinfection: Disinfection of embryo surface is unnecessary unless a systematic infection is present. Instead mature seeds entire ovules or fruits are surface sterilized and embryo removed aseptically from the surrounding tissue.
- ***Sterilization**:* • Entire Seeds are surface sterilized and seeds removal under aseptic conditions.
- There are then spread in a single layer on the surface of an agar medium using a sterile needle.
- Sterilization is carried out by immersing the material in hypo chloride.
- Containing commercial bleach (5-10% clorox,0.45% Caocl2 or NaOCl) for 10-5 minute or ethanol (70-75) for 5 minutes.
- A small amount (0.01-0.1) % of a surfactant (Tween 20, Tween 80, Teepol or Mannoxol) added to the disinfection solution to increase the tissue wettability

The seeds were washed by submerging them in water with a few drops of detergent in a beaker and shake them by hand. The embryo was teased and collected without any damage. It was washed with distilled water and then treated with 70% alcohol for 30 seconds. This was followed by rinsing completely with distilled water and then transferred to 20% sodium hypochlorite, where it was left for 0 minutes. Then the embryo was thoroughly rinsed with distilled water for 3 times and dried using the autoclaved tissue paper and inoculated in the culture tubes containing the MS medium. The culture tubes were incubated at 25oC under 16 h photoperiod for 2 to 3 weeks.

Results:

***Plant in vitro regeneration*:** Many concentrations and combinations of growth regulators were used to define an efficient regeneration medium. However, zygotic embryos in MS medium supplemented with (2.0 mg/L) BAP had the highest percentage of

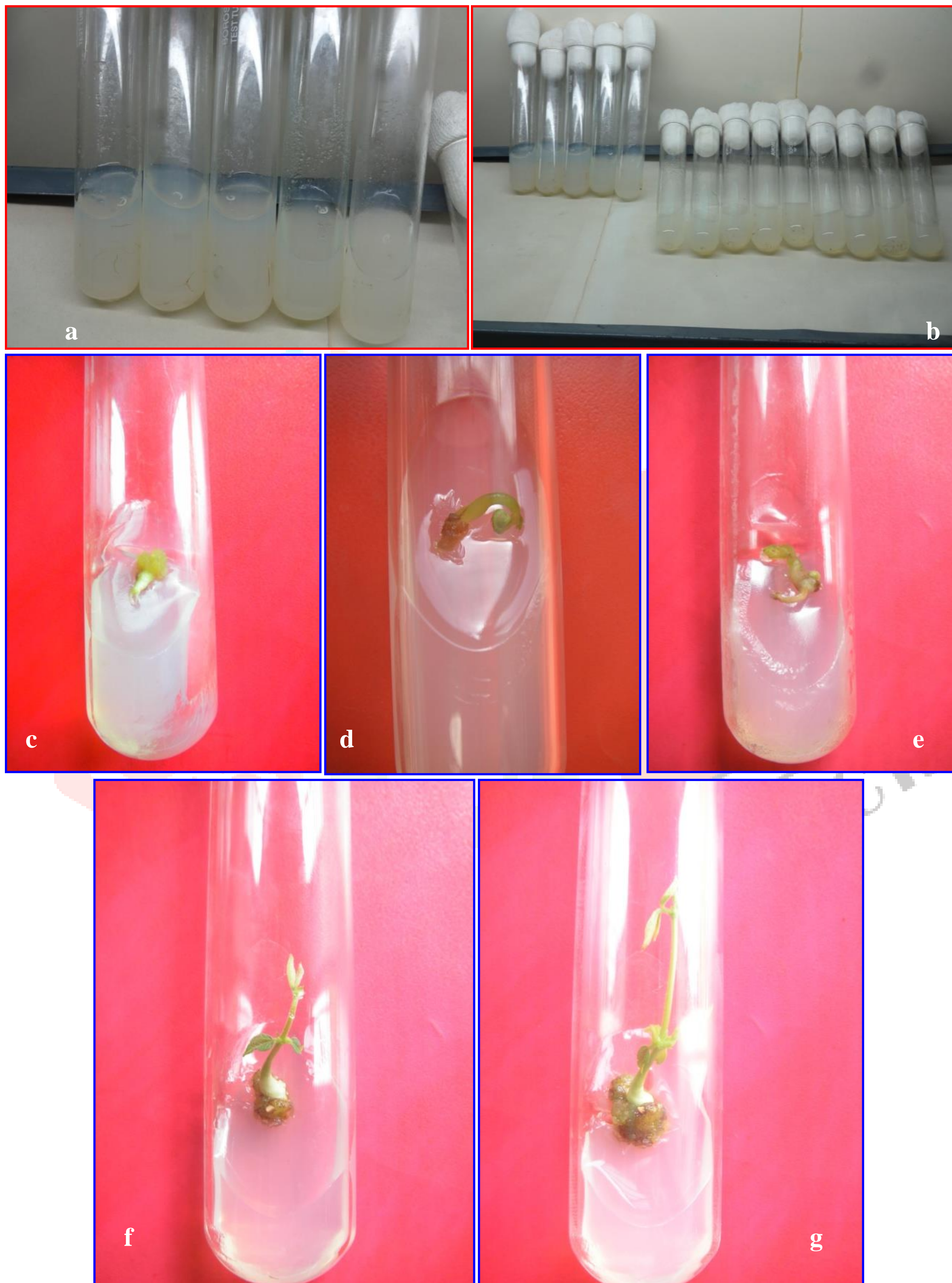

Fig-II Organ genic response of mature Zygotic Embryo culture of Green Pepper: a and b After inoculation c, d, and e after one week of cultured on MS medium supplemented with 3.0 mg/l of BAP. F after two weeks G after four weeks of culture.

Regeneration (80%), number of shoots (3.25), buds (2.85) and leaves (3.15), multiplication rate (27.75), and length of shoots (1.96 cm).

Table – 1: Composition of Plant Tissue Culture media MS (Murashige and Skoog 1962)

	Constituents	Chemical Formula	Amount in Stock (Mg/L)	Amount in Medium (Mg/L)
	(a) Macronutrients			
1	Ammonium nitrate	$NH_4)NO_3$	33,000	1650
2	Potassium nitrate	KNO_3	38,000	1900
3	Calcium-chloride	$CaCl_2\ 2H_2O$	8,800	440
4	Magnesium sulphate	$MgSO_4\ 7H_2O$	7,400	370
5	Potassium dihydrogen phosphate	$KH_2\ PO_4$		170
	(b) Micronutrients			
1	Potassium Iodide	KI		0.83
2	Boric acid	$H_3\ BO_3$		6.20
3	Manganese sulphate	$MnSO_4, 4H_2O$		22.30
4	Zinc sulphate	$ZnSO_4, 7H_2O$		8.60
5	Sodium molybolate	$Na_2\ MOO_4\ 2H_2O$		0.25
6	Copper sulphate	$CuSO_4, 5H_2O$		0.025
7	Cobalt chloride	$COCl_2\ 6H_2O$		0.025
8	Ferric sulphate	$FeSO_4, 7H_2O$		27.8
	(C) Fe (Iron) -EDTA			
1	Ferrous Sulphate Ethylene diamine tetra acetic acid disodium salt (Dihydrate)	$Fe\ So_4.7H_2O$ $Na_2EDTA.2H_2O$		37.8
	(c) Vitamins and organics			
1	Meso-Inositol	$C_6H_{12}6O_6$		100
2	Glycine (Amino acetic acid)	$C_2H_5NO_2$		2.0
3	Thiamine-HCl (Vit.B1)	$N_{12}H_{17}CIN_4.5HCl$		0.1
4	Pyridoxine-HCl (vit.B6)	$C_8H_{11}NO_3.HCl$		0.5
5	Nicotinic acid (Pyridine-3-carboxylic acid	$C_6H_5NO_2$		0.5
	(d) Sucrose			3000.0
	pH			5.6 to 5.8

Direct bud formation was observed only in zygotic embryos cultured on MS medium added with BAP. Optimum values for bud induction from zygotic embryos were obtained in MS medium, supplemented with 2 mg/l of BAP (Table 1). One hundred percent of explants cultured on this medium turned green and showed a good differentiation: cotyledons spread and became large; hypocotyls reached an average of 1 cm to 1.5 cm in length. For 6 days of culture we observed the emergence of some leaves and globular structures on the embryo hypocotyl without any intervening callus (Fig. 1). Continuous and asynchronic growth of buds was formed and the responses to organogenesis varied between cultured embryos (from 3 to 19) with a mean rate of 7.5 per organogenic explant. Fifteen-day-old organogenic embryos, with adventitious buds, were transferred to development medium to permit shoot growth. Scrubby shoots (2 leaves stage) were transferred into a third medium containing 3.0mg/l BAP which permitted their elongation. Full-grown shoots excised from hypocotyl explants were transplanted into rooting medium. The most efficient rooting medium was the

growth regulator-free MS medium at half strength. We observed that even not well elongated shoots rooted on this medium as also reported by Ebida and Hu (1993).

The histological analysis of the observed ontogeny was carried out on explants at different stages of their development. Embryos after 4 days culture showed a peripheral layer of meristematic primary cells. These structures increased gradually in size followed with bud formation. After 10 days culture on the organogenic medium, bud primordia emerged from the external layer of the hypocotyl and an apical meristem surrounded by leaves was observed.

Discussion:

In this *Capsicum annuum*. L cv CA960, we demonstrate, for the first time reporting this experiment, that hypocotyls of mature zygotic embryos of the cv CA960 pepper variety an efficient organogenic explants. However, germinated seedlings as explants failed in regeneration (Arous *et al.,* 1998). Such young tissue seems to have the best response to *in vitro* regeneration and it was successfully used by many authors in pepper (Binzel *et al.*, 1996a, Harini and Lakshmi, 1993) and other species (Bailey *et al.*, 1993, Kosturkova *et al.*, 1997).

Our results indicate that bud induction is strongly dependent on the addition of BAP in the culture medium. This cytokinin plays a crucial role on the induction of organogenesis as observed in many peppers tissue culture (Phillips and Hubstenburger, 1985, Binzel *et al.*, 1996b). However, the addition of Kin did not give the same success in bud induction from pepper zygotic embryos of var-Dh. Similar type of response was published by Pandeva and Simeonova (1992) in contrast to Agrawal *et al.* (1989) and Binzel *et al.* (1996b) who described the differentiation of bud shoots in the presence of Kin.

It is the culture of mature embryos derived from ripe seeds. This type of culture is done when embryos do not survive in vivo or become dormant for long periods of time, or, is done to eliminate inhibition of seed germination.

Conclusion:

This success of *in vitro* Mature Zygotic Embryo Culture makes the use of appropriate genetic transformation program of *Capsicum annuum* L. cv CA960 possible, particularly transformation, in order to develop a new resistant variety, especially to virus.

- Embryo culture is a valuable *in vitro* tool for breeding.
- It is most often used to rescue embryos from interspecific and intergeneric crosses and from embryos that do not fully develop naturally (as in early ripening and seedless fruit where the embryo aborts).
- The method also can be used to rescue seedless triploid embryos, produce haploids, overcome seed dormancy, or determine seed viability.
- It is useful in understanding embryo morphogenesis and precocious germination.
- As research continues with this technique, new and valuable uses will be developed to assist the biotechnological breeding of plants.

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