Plant regeneration from zygotic embryo hypocotyls of Tunisian chili (*Capsicum annuum* L.)

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Abstract

We report the regeneration of Tunisian pepper variety from zygotic embryos cultured *in vitro*. Zygotic embryos of the Tunisian cultivar D'hirat cultured *in vitro* develop adventitious buds. The best results for bud induction were obtained in Murashige and Skoog medium, supplemented with 6-benzylaminopurine (5 mg/l) and naphtaleneacetic acid (1 mg/l). Important effect of 6-benzylaminopurine in adventitious bud formation was demonstrated. Shoot bud development was enhanced by the addition of gibberellic acid to the medium. Plants were rooted in Murashige and Skoog medium at half strength and transferred into pots, containing loam. To test the stability of the regenerants, characters related to the fertility, fruit quality, leaf and flower were measured. Regenerants and their progeny were compared to control plants derived from seeds. Variance analysis and CANDISC were used in our investigation. We detected no significant differences between regenerants and control plants for the characters tested.

Key words: Pepper, *Capsicum annuum* L., *in vitro* culture, bud induction, direct neoformation, plant conformity

Introduction

The susceptibility of pepper to many pathogens, including viruses, fungi, bacteria and nematodes constitutes the main problem for the cultivation of this species. The most severe incidence and economic impact are due to viral diseases (Martinelli and Quacquarelli, 1983), which may destroy entire harvest (Caranta, 1995). Therefore, the efficient way to preserve culture could be achieved by selecting resistant varieties in order to obtain durable and respectable yields. Many programs have been established to obtain transgenic disease-resistant pepper plants. Such genetic improvement allows the rapid selection of resistant varieties and possibly combine a resistance to many pathogens. The use of transgenic plants is a method for the improvement of resistance to diseases that are unsatisfactorily controlled by chemicals. This selection is important for economical progress and will suit human needs. For gene transfer technology to be useful, however, *in vitro* regeneration of complete and fertile plants is required (Williams *et al.*, 1998).

Regeneration of pepper plants from different explants has been reported. Organogenesis from many explants has been described (Gunay and Rao, 1978, Phillips and Hubstenberger, 1985, Sripichitt *et al.*, 1987, Agrawal *et al.*, 1989, Valera-Montero and Ochoa-Alejo, 1992, Szász *et al.*, 1995). The induction of somatic embryogenesis from anthers and immature zygotic embryos was reported respectively by Sibi *et al.* (1979) and Binzel *et al.* (1996a). Recently, the successful transformation of pepper using *Agrobacterium tumefaciens* has been reported (Zhang *et al.*, 1994, Yu-Xian Zhu *et al.*, 1996, Kim *et al.*, 1997 and Kang *et al.*, 1998).

In Tunisia, *Capsicum annuum* L. occupies an important place in agriculture. Arable land reserved for this crop were 27% in 1992 (FAO, 1992) and 56% in 1996 (Ministère de l'Environnement et de l'Aménagement du Territoire, 1998). This culture is susceptible to many pathogens, making the production of resistant varieties by gene transfer an important goal.

Until now there has been no efficient *in vitro* culture system for local pepper varieties. Published regeneration protocols for pepper have not been reproduced on Tunisian varieties due to the genotype effect. This difficulty, has been noted by many authors (Szász *et al.,* 1995, Franck-Duchenne *et al.*, 1998, Mihálka *et al.,* 1998). In our study, the main purpose was to elaborate an adapted protocol of *in vitro* plant regeneration to Tunisian pepper.

Referring to previous work, tissue and cell *in vitro* culture frequently caused alterations to regenerated plant tissue affecting some characters (Evans and Sharp, 1983, Toldi *et al.*, 1996). Consequently, our second objective was to verify the qualities of our regenerants using germinated plants as controls before any transformation investigations.

Materials and methods

Plant material: Tunisian pepper, variety "D'hirat" (var-Dh), was used in our study. It was selected by the Institut National de Recherche Agronomique de Tunis. This variety is sensitive to viruses mainly PVMV, CMV and PVY (Khadmaoui, 1996). var-Dh 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

obtained by selfing. Mature seeds 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 plate containing culture medium (5 or 6 embryos per plate).

Tissue culture: The MS basal culture medium we used comprised the inorganic and organic nutrients of Murashige and Skoog (1962) and was supplemented with 3% sucrose and solidified with 0.8% agar.

Naphtaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4D) and 3-indoleacetic acid (IAA) as auxins and 6-benzylaminopurine (BAP) and kinetin (Kin) as cytokinins were tested in our experiments. The concentrations of auxins varied from 0.5 mg/l to 2 mg/l and that of cytokinins ranged from 1 mg/l to 6 mg/l.

The pH of the medium was adjusted at 5.8 before autoclaving at 120°C for 20 min.

Cultures were placed in a culture room under a controlled temperature (27 ±1°C) with a 16-h photoperiod. After 2 weeks in the organogenic medium containing 5mg/l BAP and 1mg/l NAA, cultures were transferred to a development medium with a lower concentration of growth regulators (3 mg/l BAP and 0.5 mg/l NAA). Buds, with slow growth, were subcultured in an elongation medium, containing 1mg/l BAP and 0.5 mg/l NAA. In combination to BAP and NAA, 0.5 mg/l GA_3 (Gibberellic acid) was added to the second and third media. Well developed shoots were transferred to MS rooting medium devoid of growth regulators and at half strength. Each treatment was repeated three times using 30 embryos.

Histological studies: The histological observations were carried out to determine the ontogeny of shoot buds. Explants, at different stages of their development, were fixed in a 3:1 mixture of ethanol and acetic acid for 24 hours. The tissues were then dehydrated in ethanol and xylene. The samples, embedded in paraffin, were sectioned with a microtome at 7 µm. The sections were thoroughly deparaffinized, stained with hematoxylin and safranin and finally examined by a microscope.

Data analysis: Forty-six regenerated plants (R_0) and 24 of their progeny (R_1), obtained by selfing, were grown in the same conditions of control plants from 25 seedlings (T). All the analysed plants were obtained from the same self-fertilized plant of var-Dh.

To compare the three groups of plants, 12 characters were considered: number of swollen ovaries (V1), from the 30 first flowers to open out, length (V2) and width (V3) of the leaf axilling the first flower to appear, mean length (V4) and weight (V5) of ten randomly chosen fruit, mean seed number (V6) and weight (V10) per fruit, weight was measured for 20 seeds of each fruit, mean diameter (V7) of an open flower, mean length (V8) and width (V9) of their petals, number of viable (V11) and fertile pollen grains (V12). Ten flowers were chosen from each plant. The pollen was stained in Alexander solution: the viable pollen fixed the colorant and became red. The fertility of the pollen was considered on the germination of the pollen tube induced on 20% sucrose solution. The total number of pollen grains analysed was 500 for each plant.

For examining the relationship among quantitative variables of the three groups, the analysis of variance models (ANOVA); *F* test was chosen. The mean comparison for each variable was carried out by the Duncan multiple range test. Statistical significance was determined at the 0.05 probability level.

To examine R_0, R_1 and T plants, considering all the variables, treatment by multivariate technique, a canonical discriminant analysis (CANDISC) was conducted. Data were analysed using different SAS program procedures (1989).

Results

Plant *in vitro* regeneration: Many concentrations and combinations of growth regulators were used to define an efficient regeneration medium. Direct bud formation was observed only in zygotic embryos cultured on MS medium added with BAP. The combination of BAP and NAA increased the percentage of organogenesis and the development of the explants. While, in MS medium, added with NAA alone we obtained the development of the embryos to a complete plant and no adventitious buds were observed.

Substitution of NAA by 2,4D or IAA was not active in a medium added by BAP. Combination of 2,4D and BAP initiated callogenesis without bud formation and the combination of IAA and BAP stimulated the germination of the embryos. Embryos also failed to respond to the Kin combined with NAA, 2,4D or IAA.

Optimum values for bud induction from zygotic embryos were obtained in MS medium, supplemented with 5 mg/l of BAP and 1 mg/l of NAA (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. Since 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. Addition of GA_3 was optimal for both continuous growth and elongation of buds (Fig. 2). Control medium devoid of GA_3 was less active and 60% of shoots turned yellow and withered.

Scrubby shoots (2 leaves stage) were transferred into a third medium containing 1mg/l BAP, 0.5 mg/l NAA and 0.5 mg/l GA_3 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 (Fig. 3). We observed that even not well elongated shoots rooted on this medium as also reported by Ebida and Hu (1993).

Table 1. Effect of BAP and NAA concentration on the percentage of organogenic embryos (O.E.) and mean number of shoots per embryo (NBSE) in var-Dh.

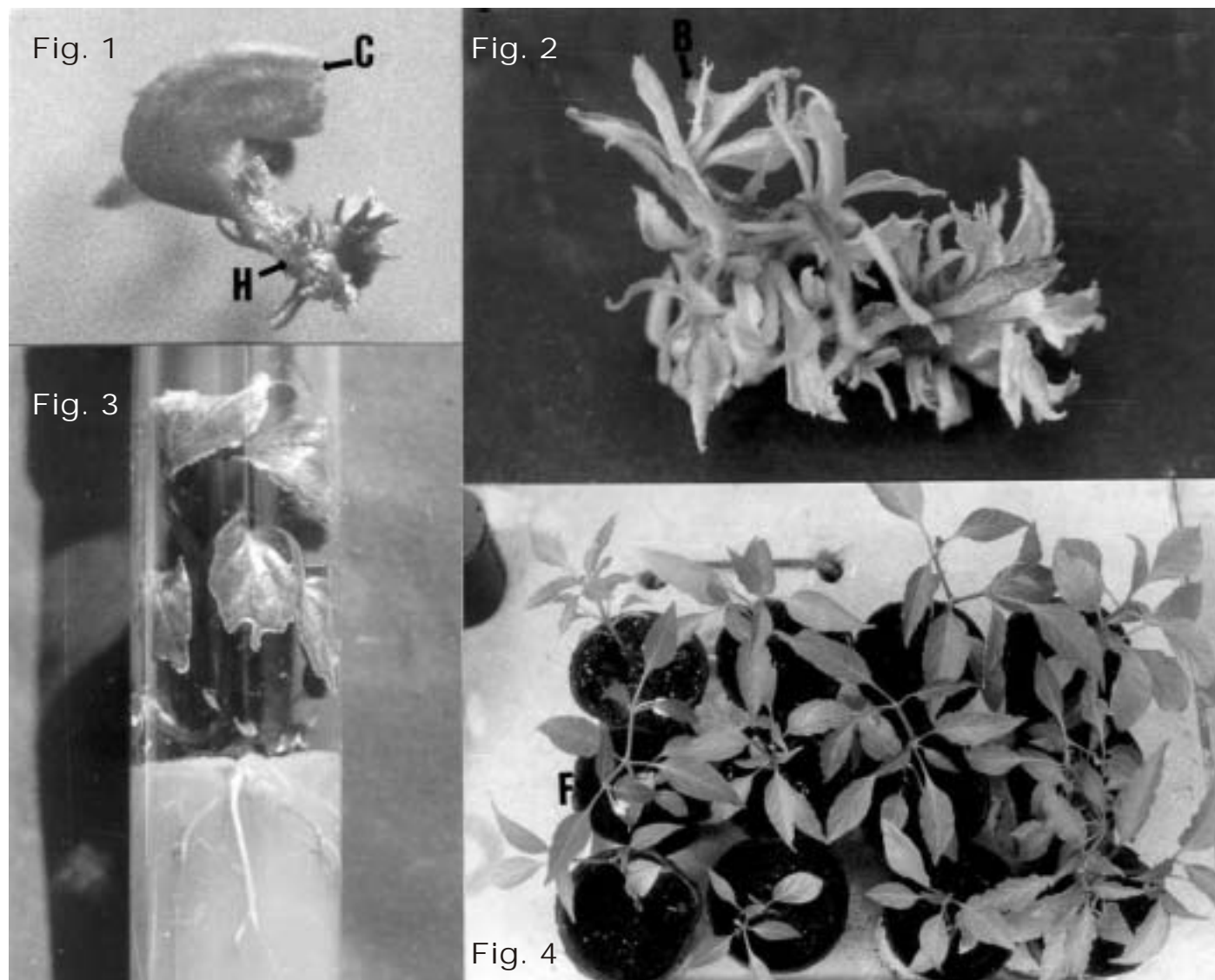| NAA (mg l^{-1})	BAP (mg l^{-1})	O.E. (%)	NBSE
1	0	0 ±0	0 ±0
0	5	54.6b (± 8.7)	3.10c (± 0.65)
0.5	5	60.0a (± 8.3)	3.33c (± 2.03)
0.5	6	70.0a (± 7.2)	4.70b (± 1.89)
1	2	20.0c (± 6.8)	1.20c (± 0.43)
1	5	77.4a (± 4.2)	7.55a (± 2.46)
1	6	66.5a (± 7.6)	3.66c (± 1.76)
2	5	71.4a (± 6.0)	2.25d (± 1.15)

The average designated with the same letter are not significantly different.

Regenerated plants with six or eight leaves were potted protected by gaze and kept about one week in the culture room. Subsequently, they were placed in a surrounding (ambient) culture room atmosphere. Surviving plants, nearly 100%, were transferred to the greenhouse and grown to maturation (Fig. 4). Selfing were obtained from these regenerated plants.
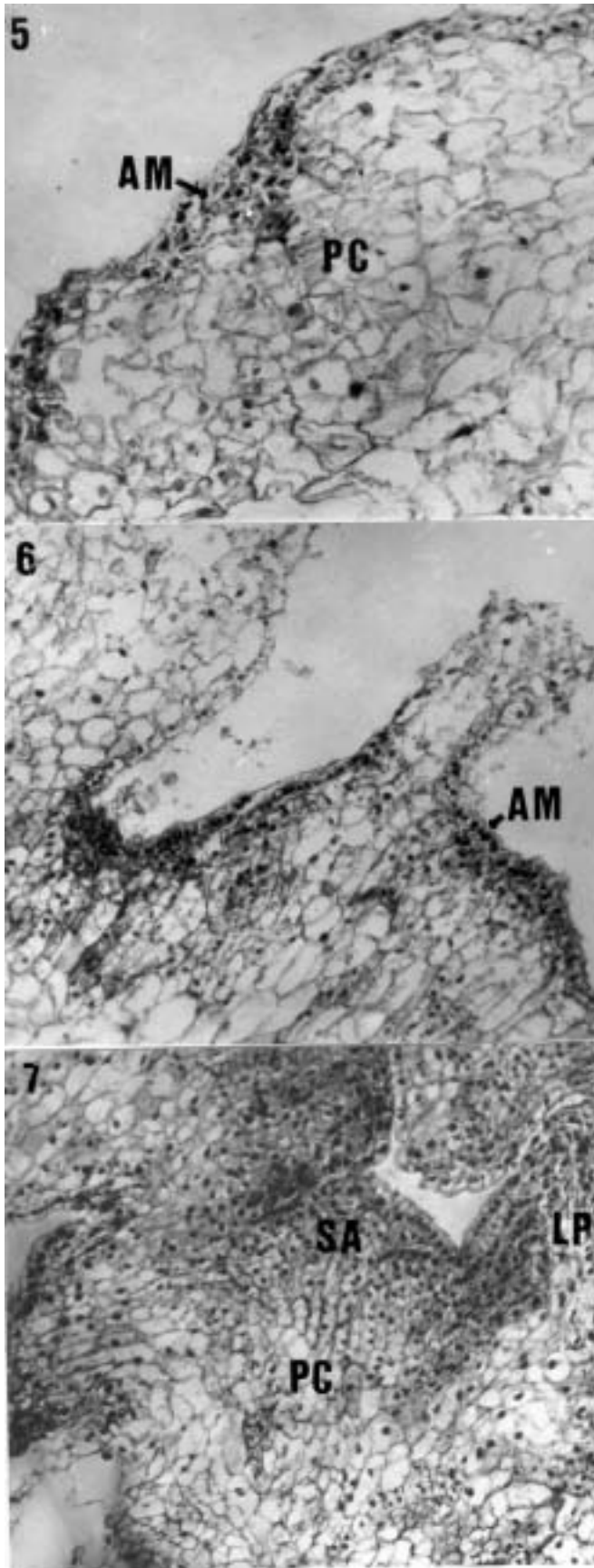
The histological analysis of the observed ontogeny was carried out on explants at different stages of their development. Embryos after 4 days culture showed a peripheral layer of meristematic primary cells (Fig. 5). These structures increased gradually in size followed with bud formation. After 10 days culture on the organogenic medium (Fig. 6), bud primordia emerged from the external layer of the hypocotyl and an apical meristem surrounded by leaves was observed (Fig. 7).

Genetic stability: The stability of the regenerants and their progeny was statistically analysed on 12 characters measured for R_0, R_1 and T plants.

The variance analysis revealed significant differences for three variables: the leaf width (V3), the flower diameter (V7) and the petal length (V8) (Table 2). The Duncan's test for these three variables demonstrated a higher average for the T group (Table 3). In other way, differences between T and R_1 on one side and R_1 and R_0 in the other side are not significant for the variables V7 and V8, but the differences between T and R_0 are significant. The V3 revealed a significant difference between the group R_0 and R_1 and the group formed by T plants.

Fig. 1. Organogenic embryos 10 days cultured on MS medium supplemented with 5 mg/l of BAP and 1 mg/l of NAA. C: Cotyledon, H: Hypocotyl. **Fig. 2.** Organogenic embryo after 3 weeks culture on. B: Bud. **Fig. 3.** Root induction. **Fig. 4.** Entire and fertile regenerated plants transferred in pots. F- Flower.

For the other variables, like fruit characters and plant fertility, the *in vitro* culture effect was not significant.

Table 2. ANOVA analysis: results of the *F* test

Variables	MS	*F*	Significance
V1	6.294	2.01	NS
V2	7.224	3.08	NS
V3	2.196	5.72	S
V4	1.677	2.75	NS
V5	0.100	0.04	NS
V6	2038.566	2.66	NS
V7	0.241	5.02	S
V8	0.071	5.4	S
V9	0.051	2.87	NS
V10	674.649	1.34	NS
V11	6.294	0.09	NS
V12	290.100	0.23	NS

MS: Mean Square, S: significant difference, NS: non significant difference at the p=0.05 level.

Table 3. Mean comparison by DMRT test

Variables	R_0	R_1	T
V3	3.77b	3.75b	4.252b
V7	2.09b	2.17ab	2.26a
V8	1.14b	1.18ab	1.24a

The average designated with the same letter (a or b) are not significantly different.

Table 4. Eigenvalues and composition of canonical structure Can1 and Can2

Can	Proportion	Cumulative	Canonical structure			
Can1	0.695	0.695	V2	V3	V4	V8
Can2	0.304	0.100	V1	V6	V7	V10

The CANDISC (Fig. 8) demonstrated no separation between the groups. The maximum of variability was explained by the first canonical component which includes 69% of the variability (Table 4). It was correlated with the variables, V2, V3, V4 and V8. No clear separation was detected.

Discussion

In this paper, we demonstrate, for the first time, that hypocotyls of mature zygotic embryos of the Tunisian pepper variety "Dh" are an efficient organogenic explant. 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 pepper tissue culture (Phillips and Hubstenburger, 1985, Binzel *et al.*, 1996b). However, the addition of Kin did not gave 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.

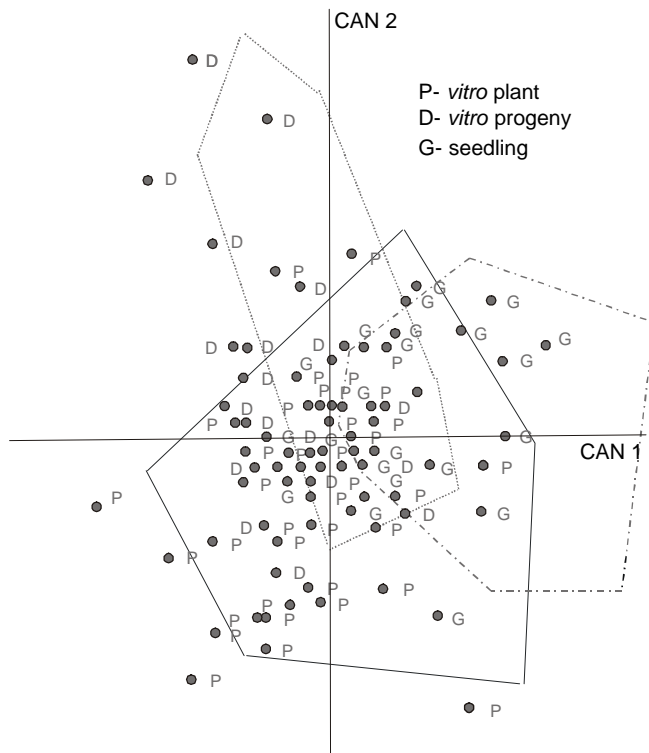The best response was observed when MS medium was

Fig. 5. Section of the embryo hypocotyl showing the superficial meristematic layer. AM: Apical meristem - PC : Parenchymatic cells.
Fig. 6. Section of the embryo hypocotyl after 10 days culture. An early stage of the development of the apical meristem.
Fig. 7. Development of the shoot bud in about 2 weeks cultured embryo. SA: Shoot apex - LP: Leaf primordia.

Fig. 8: Canonical discriminant analysis

supplemented with BAP (5 mg/l) - NAA (1 mg/l) combination. Contrarily to other auxins tested (2,4D and IAA), adding NAA to the BAP containing medium, permitted the increase of the number of buds per explant. 2,4D and IAA in combination with BAP were not effective and inhibited bud induction. According to many authors, 2,4D (Binzel *et al.*, 1996a) and IAA (Gunay and Rao, 1978, Ochoa-Alejo and Garcia-Bautista, 1990, Christopher and Rajam, 1996) were effective on pepper tissues. But on var-Dh embryos we did not obtain any neoformation.

For differentiation and elongation of shoot buds, the effect of GA_3 was noted and it is fundamental for the good continuous growth of *in vitro* regenerated buds. Many previous observations elucidate the role of the GA_3 on the elongation of shoots in pepper (Szász *et al.*, 1995) and also in other species (Nikolié *et al.*, 1997).

Referring to the literature, the somaclonal variations were increased by condition culture, cal phase, the duration of the non morphogeneic stage and the growth regulators (Shepard *et al.*, 1980, Hartman *et al.*, 1984, Evans *et al.*, 1987). To initiate transformation experiments we had to verify the conformity of the regenerants. Statistical analysis of some characters of the regenerants, their progeny and the control germination plants, revealed variations in three traits: leaf width, flower and petal length, but no consequences on the alteration on fruit characters or fertility were observed. Decrease of the regenerants leaf width, compared to their progeny and control plants was probably due to the effect of *in vitro* culture conditions, especially the influence of the growth regulators. Epigenetic effects of the artificial culture phase of the *in vitro* plants probably masked true genetic factors and was not transmitted to the progeny. Change on leaf shape was observed by Ogihara (1981) in the regenerants of *Haworthia* but no consequences were detected.

The statistical analysis, to evaluate the stability of regenerated plants, revealed that the regenerants maintain the main cultivar agronomic characters. In present investigation, *in vitro* regeneration protocol the direct regeneration and the rapid neoformations (about 6 days culture) were beneficial for the stability of *in vitro* plants. The stability of pepper regenerants, was also verified by Agrawal *et al.* (1989); this stability may be the consequence of direct differentiation of shoot buds (Agrawal *et al.*, 1989).

This success of *in vitro* regeneration makes the use of appropriate genetic transformaïon program of *Capsicum annuum* L. possible, particularly for var-Dh transformation, in order to develop a new resistant variety, especially to virus.

The neoformation from external surfaces of the explant, demonstrated by histological study, will increase the success of a transformation-regeneration protocol (Torregrosa, 1994) and the direct regeneration may be positive for the stability of new genetic information in any transgenic plant.

Acknowledgement

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