

Micropropagation of *Crambe Abyssinica* Hochst. Ex R.E. Fr.

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Abstract

Crambe abyssinica Hochst. Ex. R. E. Fr. is an oilseed species that produces non-edible oil, initially employed in Brazil as a culture for soil surface coverage and animal feed. However, with stimulation of the production and use of renewable energies, it arose as an interesting alternative for biodiesel production. The aim of this work was to describe a protocol for micropropagation of crambe, using apical segments as explants. Crambe seeds germinated *in vitro* in medium MS½ provided apical segments adequate to the micropropagation process. In the shoot induction phase, testing the interaction of the cytokinins 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ) as well as different concentrations thereof, the highest numbers of shoots per explant were obtained with 5 µM BAP at 30 days (9.26 shoots) and 15 µM BAP at 60 days (18.08 shoots) of *in vitro* culture. In the elongation with 1 µM gibberellic acid (GA₃), the mean length of the shoots turned from 3.61 cm to 5.03 cm after 30 days. The highest percentage of rooting was of 30% with 0.25 µM naphthalene-acetic acid (NAA). The results of this work revealed a satisfactory frequency of shoot regeneration from apical segments of crambe in media containing 5 µM BAP, and adequate elongation with 1 µM GA₃. Nevertheless, there is still need for efficient rooting and acclimatization for complete *in vitro* propagation of crambe.

Keywords: cytokinins; *in vitro*; oilseeds; organogenesis; regeneration.

Introduction

Crambe abyssinica Hochst. Ex. R. E. Fr. (Brassicaceae) is an oilseed species that produce non-edible oils for biodiesel production. Originated in Ethiopia and domesticated in the Mediterranean region (Knights, 2002), this species was introduced in Brazil in the state of Mato Grosso do Sul (Dahlke and Simonetti, 2010). It was initially employed as a culture for soil surface coverage and animal feed (30 to 32% of crude protein) (Neves et al., 2007), but with the stimulation of the production and use of renewable energies, crambe arose as an interesting alternative also for biodiesel production (Roscoe et al., 2007).

Other advantages of the cultivation of crambe refers to its rapid cycle (Chhikara et al., 2012), high biomass production (Zulfiqar et al., 2011), elevated amount of seeds (Falasca et al., 2010), low production cost in relation to other oilseeds (Jasper et al., 2010), resistance to insects (Kmec et al., 1998), percentage of total oil in the seed (32 to 38%), which surpasses that of soybean (Falasca et al., 2010), and elevated percentage of erucic acid in the oil extracted from the seeds (50-60%), useful in the plastic and lubricant industry (Lara-Fiozeza et al., 2013). Moreover, the culture has potential for phytoremediation against heavy metals (Paul et al., 2010).

There is great interest in the breeding of cultivated species with the aim of increasing productivity. For this, genetic transformation and *in vitro* culture techniques are important tools to be employed. However, the genetic transformation of plants demands the successful application of these techniques, requiring an efficient, viable, genetically loyal propagation system that provides plant material of good quality to satisfy this demand (Memon et al., 2009).

According to Li et al. (2011), the development of an efficient protocol for the regeneration of crambe cultured *in vitro*, by means of tissue culture techniques, becomes relevant to improve the quality and production of oil in the species.

With regards to the micropropagation, different parts of the plant have been used as explant, for instance hypocotyls (Li et al., 2010, 2011; Palmer and Keller, 2011; Chhikara et al., 2012), cotyledons (Palmer and Keller, 2011; Chhikara et al., 2012), root (Palmer and Keller, 2011; Chhikara et al., 2012) and cells in culture (protoplasts) (Gao et al., 1998), with only the protocol developed by Li et al. (2011) obtaining a satisfactory rate of shoot regeneration, of 95%. Although a great number of genetically identical plants can be regenerated by means of micropropagation, some somaclonal variations can be detected (Miguel and Marum, 2011; Pathak

and Dhawan, 2012), which in turn depend on various factors, among which the type of explant used should be considered (Leela et al., 2011). Cultivation *in vitro* from explants with meristematic regions, such as apical and nodal segments, are very popular and important (Zoghalmi et al., 2012), as the frequency of somaclonal variations in the regenerated plants is low (Singh et al., 2012). Along these lines, the aim of the present work was to describe a protocol for micropropagation of crambe using apical segments as explants.

Results and discussion

Shoot multiplication

The seeds of crambe germinated *in vitro* developed healthy plants (Fig 1A) suitable as sources of apical segments to be used as explants (Fig 1B), and these were established and developed adequately *in vitro* (Fig 1-C-E). In general, the results evidence that the used cytokinins efficiently and satisfactorily promoted the multiplication of shoots from apical segments of crambe. The medium MS without cytokinins (0 μM) produced numerous roots at 30 days and the apical dominance was not affected, but at 60 days senescence and death of most of the plantlets was noticed (data not shown). The same results were reported for apical segments of *Raphanus sativus* var. *radicula* Pers. (Pavlović et al., 2012). This demonstrates the importance of cytokinins in the suppression of apical dominance, resulting in ample development of new shoots and inhibition of root appearance. In the phase of shoot induction, the analysis of variance indicated that there was significant interaction between the cytokinins (BAP, KIN and TDZ) and the concentrations (0, 5, 10, 15 and 20 μM) only for the variables number of shoots per explant (NSE) at 60 days. This suggests the existence of differentiated response in the multiplication of shoots in relation to the dosages of each cytokinin after a longer period of contact with the explant (Table 1).

For NSE, at 30 days, the analysis of variance showed that only the types of cytokinin were significant (Table 1), and that among the tested cytokinins, BAP obtained the highest value (9.26 shoots), although not differing significantly from TDZ (8.23 shoots) (Table 2). At 60 days, the significant interaction between the factors demonstrated that, in all tested concentrations, BAP presented values superior to the other compounds with a significant difference, being that at the concentration of 15 μM the highest value for NSE (18.08 shoots) was detected (Table 3). In both evaluation times, TDZ was more efficient than KIN in inducing shoots in most of the concentrations, differing statistically only at 60 days with 5 μM (Tables 2 and 3). It is important to notice that there was considerable increment in NSE from 30 days (general mean: 8.69) to 60 days (general mean: 12.98), indicating that until this time point the cytokinins acted in a regular way.

The analysis of regression for the cytokinin concentrations by NSE at 30 and 60 days is presented in figure 2. It can be verified that the response was quadratic, except for KIN at 60 days, which was positive linear. By the regression equation of BAP, at 30 days, with a concentration of 12.23 μM it would be possible to obtain a maximum value of 10.62 shoots, and at 60 days and 13.46 μM , this number might reach 18.74 shoots.

BAP is one of the cytokinins with lowest cost, and has been very efficacious in the multiplication of diverse Brassicaceae species (Shukla and Sawhney, 1991; Memon et al., 2009; Ishigaki et al., 2009). Studies related to induction of shoots and regeneration of whole plants from apical segments have

not yet been described for crambe. However, previously published protocols for *in vitro* regeneration were obtained by using hypocotyls as explants, enabling studies related to the genetic breeding of the species, such as transgenesis and somatic hybridization (Wang and Luo, 1998; Wang et al., 2003, 2004).

Li et al. (2010), working with hypocotyls as explants, described a protocol for direct induction of shoots in medium MS supplemented with 10 μM TDZ and 2.7 μM NAA, promoting a regeneration frequency of up to 60% for *C. abyssinica* cv. *Galactica*. In a new work, Li et al. (2011) obtained 95% of shoot regeneration using the medium Lepiovre, also supplemented with 10 μM TDZ and 2.7 μM NAA. Chhikara et al. (2012) noted efficient regeneration in medium MS with 8.8 μM BAP and 0.10 μM NAA, with a frequency of 70%, starting from calli of hypocotyl. In this work, although a different explant was used than in the above-cited works, the applied concentrations of cytokinin were close, and efficiently promoted the expected morphogenetic response both with BAP as well as with TDZ. All the results above corroborate the fact that, just as for the Brassicaceae species, also for crambe the value of 5.0 to 10 μM BAP is ideal for *in vitro* regeneration of shoots, and that this regulator at these concentrations has been one of the most used for this family.

Shoots elongation

The shoots of crambe that presented better morphological aspect and development in the phase of shoot induction, regenerated from the treatments with BAP and TDZ, besides the control, were selected for the elongation phase. All shoots obtained through these treatments were transferred into medium MS supplemented with a single concentration (1 μM) of GA_3 , and were kept under these conditions for 30 days. The analysis of variance in this phase demonstrated that there was no significant interaction between the cytokinins and the concentrations for the MLS (Table 4). This indicates that the influence of GA_3 on shoot elongation was independent from the cytokinins and concentrations previously applied in the phase of shoot induction. Besides, this response on the elongation can be verified when comparing the previous general mean value, which was of 3.61 cm at 60 days; after 30 days with presence of GA_3 in the medium, an overall MLS of 5.03 cm was obtained (Table 4). The GA_3 added to the culture medium may promote increase in shoot length due to stimulation of cell division and elongation (Rocha et al., 2009). The incorporation of 1 μM GA_3 produced shoots with overall mean length considered adequate to perform the following steps of the micropropagation, which are the rooting and subsequent acclimatization of the plants. Shoots with small length may show low percentage of rooting if cultivated directly in rooting media, or originate saplings with low quality for the acclimatization phase (Rocha et al., 2009).

Purkayastha et al. (2010) reported that 0.5 μM GA_3 increased threefold the elongation in 98% of the cultures. The elongation of atrophied shoots, generated in medium containing cytokinins for elevated times and concentrations, is dependent on the reduction or absence of the cytokinins or incorporation of GA_3 to the medium (Paul et al., 2010). This effect of GA_3 in relation to BAP has also been reported in other plant species (Sugla et al., 2007; Purkayastha et al., 2008). Chhikara et al. (2012) mentioned that, to induce the development of adventitious roots in crambe *in vitro*, it is necessary to incorporate GA_3 into the medium.

Table 1. Summary of the analysis of variance for the number of shoots per explant (NSE) and percentage of survival (% Surv.) of *C. abyssinica* plantlets under interaction of different concentrations of BAP, KIN and TDZ after 30 and 60 days of culture *in vitro* in MS medium.

FV	DF	F-Test			
		NSE		%Surv.	
		30 d	60 d	30 d	60 d
Cytokinin (A)	2	5.05**	56.42**	0.71 ^{ns}	1.50 ^{ns}
Concentration (B)	4	18.79 [~]	32.15 [~]	1.52 [~]	30.71 [~]
Int. A x B	8	1.20 ^{ns}	6.46**	0.37 ^{ns}	1.10 ^{ns}
Residue	60	-	-	-	-
General mean	-	8.69	12.98	96.95	91.72
CV (%)	-	13.41	12.11	6.58	6.46

** , * significant at 0.01 and 0.05 probability, respectively; ns: non-significant; CV(%): coefficient of variation in percentage.

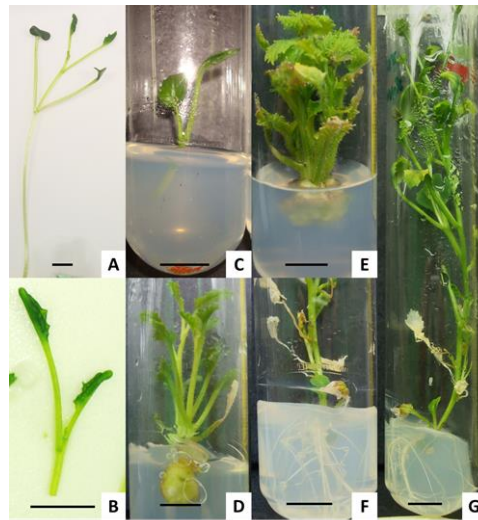


Fig 1. Regeneration of *C. abyssinica* through apical segments in MS medium. (A) Plant germinated *in vitro*; (B and C) Apical segment used as explant (0 days); (D and E) Shoots induced with 5.0 µM BAP at 30 and 60 days, respectively; (F and G) Rooting of shoots with 0.5 µM NAA at 120 days. Bar = 1.0 cm.

Table 2. Mean number of shoots per explant (NSE) of *C. abyssinica* plantlets under different cytokinins after 30 days of culture *in vitro* in MS medium.

Cytokinins	Mean
BAP	9.26 a
KIN	8.23 b
TDZ	8.58 ab

There is no difference between means followed by the same letter by Tukey test at 5%.

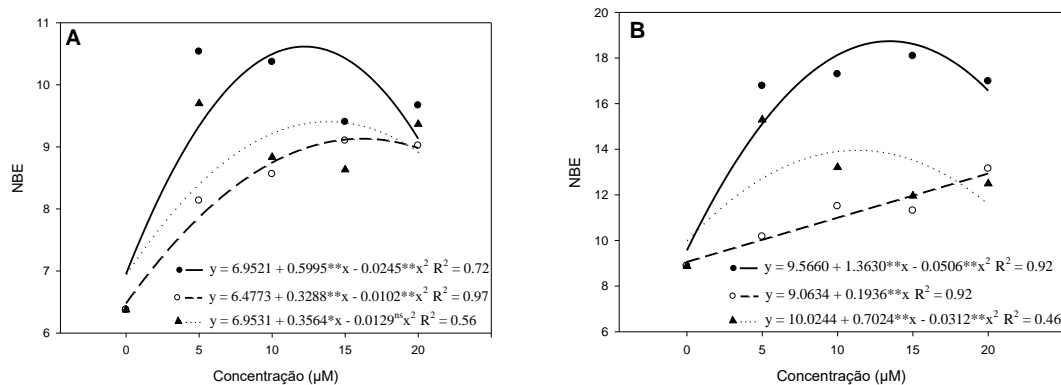


Fig 2. Polynomial regression of the mean values for the number of shoots per explant (NSE) of *C. abyssinica* plantlets subjected to different concentrations of cytokinins after 30 days (A) and 60 days (B) of culture *in vitro* in MS medium. (●)-BAP; (○)-KIN; (▲)-TDZ).

Table 3. Mean number of shoots per explant (NSE) of *C. abyssinica* plantlets under interaction of different cytokinins and concentrations after 60 days of culture *in vitro* in MS medium.

Cytokinins	Concentration (μM)				
	0	5	10	15	20
BAP	8.87 a	16.77 a	17.28 a	18.08 a	16.97 a
KIN	8.87 a	10.16 b	11.50 b	11.30 b	13.14 b
TDZ	8.87 a	15.29 a	13.21 b	11.95 b	12.49 b

There is no difference between means followed by the same letter in the column by Tukey test at 5%.

Table 4. Summary of the analysis of variance for mean length of shoots (MLS) (cm) and survival percentage (%Surv.) in *C. abyssinica* plantlets derived from treatments with BAP and TDZ, subjected to elongation with 1 μM GA3 at 90 days of culture *in vitro* in MS medium.

FV	DF	F-Test	
		MLS	%Surv.
Cytokinin (A)	1	1.23 ^{ns}	0.18 ^{ns}
Concentration (B)	4	8.81 ^{**}	5.02 ^{**}
Int. A x B	4	0.37 ^{ns}	1.80 ^{ns}
Residue	40	-	-
General mean	-	5.03	90.68
CV (%)	-	12.67	6.90

** , * significant at 0.01 and 0.05 probability, respectively; ns: non-significant; CV(%): coefficient of variation in percentage.

Table 5. Summary of the analysis of variation for percentage of rooting (%Root.) and of survival (%Surv.) in *C. abyssinica* plantlets derived from treatments with BAP and TDZ at 5 μM , subjected to rooting with NAA and IAA at the concentrations of 0.0, 0.25 and 0.50 μM , at 120 days of culture *in vitro* in MS medium.

FV	DF	F-Test	
		%Root.	%Surv.
Cytokinin (A)	1	7.53 ^{**}	0.94 ^{ns}
Auxin (B)	4	1.76 ^{ns}	1.04 ^{ns}
Int. A x B	4	1.17 ^{ns}	1.60 ^{ns}
Residue	40	-	-
General mean	-	13.66	88.00
CV (%)	-	15.86	22.09

** , * significant at 0.01 and 0.05 probability, respectively; ns: non-significant; CV(%): coefficient of variation in percentage.

Root induction

According to Purkayastha et al. (2010), the prolonged cultivation in medium containing this regulator yields thin and excessively elongated plants, inadequate for rooting. This may also explain the low percentage of rooting obtained in the present work. The elongated shoots arising from the treatments with 5 μM BAP and 5 μM TDZ were transferred into root induction media with NAA and IAA. After 30 days in these conditions, the shoots responded to this stimulus (Fig 1–F and G). Nonetheless, there was low yield in these treatments, with an overall mean of 13.66% of rooting. The analysis of variation for the interaction between the concentrations of NAA and IAA (Table 5) did not demonstrate significance for % Root. Numerically, the highest percentage of rooting was of 30%, detected in the treatment with 0.25 μM NAA in plantlets originated from 5 μM BAP (data not shown). The results of the analysis of variance indicated difference only in the tested cytokinins, in which plantlets obtained from 5 μM BAP presented general mean value higher than 20.66% of rooting, compared to 6.66% when subjected to 5 μM TDZ.

The phenomena involved in rooting are difficult to identify, owing to their complexity, thus constituting an obstacle for adequate knowledge of this stage (Assis and Teixeira, 1998). The low rooting found for crambe may be related to the residual effects of the cytokinins and gibberellin inserted in the previous phases. According to Grattapaglia and Machado (1998), the addition of gibberellins to the medium is unnecessary and may be harmful to rooting, whereas

cytokinins added to the medium may cause its inhibition (Grimaldi et al., 2008). It was verified that explants of species with rooting difficulty have a high concentration of endogenous cytokinin, whereas species with facility to root have low concentrations of it (Hartmann et al., 1990).

The capacity of producing roots and shoots depends on the proportion cytokinin/auxin in the culture medium (Shukla and Sawhney, 1991). A higher value of this relationship favors the formation of shoots, while a lower value favors the formation of roots (Skoog and Miller, 1957; Taiz and Zeiger, 2006; Rout et al., 2006); besides, residual effects should be taken into account. The great difficulty hereby lies in measuring the residues of growth regulators during the micropropagation phases. According to Flores et al. (2009), an alternative to minimize this problem is the transfer of shoots into a nourishing medium free of growth regulators. With regards to the higher inhibitory effect reported for TDZ in the regeneration of crambe roots, it has also been described for other species (Khawar et al., 2004; Flores et al., 2007). This is explained by the fact that TDZ alters the metabolism of natural cytokinins, culminating in increase in the endogenous content of cytokinins (Mok et al., 1987, apud Flores et al., 2009).

Some works report rooting of crambe shoots from hypocotyls used as explants. Li et al. (2010) reached 59 to 67% of rooting with 0.54 and 0.27 μM NAA, respectively, in shoots from treatment with 10 μM TDZ and 2.7 μM NAA. These authors also relate that 0.49 μM and 0.25 μM IBA caused low frequencies of rooting, of 24 and 18%, respectively. In turn, Chhikara et al. (2012) induced roots

using 9.5 μM IBA in shoots obtained with 8.88 μM BAP and 0.10 μM NAA, but did not reveal the efficiency of this treatment. Wang and Luo (1998) achieved satisfactory rooting in crambe shoots generated from immature ovary after 15 days with medium MS $\frac{1}{2}$ + 0.28 μM IBA. Perhaps the long culture time and the residues of growth regulators were responsible for the low rooting seen in the present work. Success in the rooting of the explants is a prerequisite for any micropropagation protocol, as it is important in order to facilitate the establishment of the plant in the acclimatization process (Pati et al., 2006).

Acclimatization

In acclimatization, the survival rate during the pre-acclimatization process was satisfactory, but the procedure of perforating and removing the PVC film from the flasks and maintaining these conditions for 15 days was an inducing factor for the appearance of fungus in some culture media. When transferred onto the commercial substrate and soil under environmental conditions, the plantlets did not support such stress and suffered senescence, leading to death of all plantlets after 15 days. Some plants originated through the rooting *in vitro*, though being well rooted, including branched roots, did not survive when transplanted into *ex vitro* conditions (Tibola et al., 2004). Even plants with normal aspect may suffer and/or not survive this phase owing to the stress afflicted to the plants by the sudden change in relative humidity that occurs in the passage from *in vitro* to *ex vitro* conditions (Apóstolo et al., 2005). Other limiting factors in this phase occur in function of the plants under *ex vitro* conditions passing into an autotrophic state. This requires performance of photosynthesis to survive, as well as need to rapidly increment the absorption of salts, thus being at risk of attack by microorganisms (Kadlec et al., 2001).

In relation to crambe, few micropropagation protocols describe the acclimatization process (Li et al., 2010, 2011; Chhikara et al., 2012). However, Palmer and Keller (2011) registered 70% of survival during acclimatization, though not disclosing the adopted methodology.

Micropropagation can be affected by various factors, such as the age of the explant, nutrients, explant orientation, types and concentration of growth regulators, and also the time of culture and subculture (Shukla and Sawhney, 1991). Taking all these factors into account, the culture time and the longer period under influence of growth regulators may have affected the micropropagation process in this study.

Materials and methods

Plant material

The experiment was conducted at the Laboratory of Plant Tissue Culture of the Center for Agronomic Sciences, Federal University of Espírito Santo, ES – Brazil. Seeds of *C. abyssinica* from the cultivar FMS brilliant, harvest of 2011, obtained from the Fundação MS, located in Maracaju, Mato Grosso do Sul – Brazil, were used for the establishment of the *in vitro* culture.

Explant source

Apical segments were excised from crambe plantlets with 30 days age originated from germination of seeds *in vitro*. The seeds were initially washed in running water with neutral detergent. Under aseptic conditions in laminar flow hood, the seeds were then disinfested by immersion in antibiotic

solution of penicillin (10 mg L⁻¹) and rifampicin (10 mg L⁻¹) for 30 minutes (min), 70% alcohol for 1 min, followed by commercially available sodium hypochlorite 50% (active chlorine: 2-2.5%) for 30 min, and washed three times in autoclaved distilled water.

Culture medium and culture conditions

The culture medium used in the *in vitro* germination of crambe seeds was MS $\frac{1}{2}$ (Murashige and Skoog, 1962). After germination, apical segments, with approximately 2-3 cm, were obtained and used in the micropropagation process from plantlets with 30 days age. For induction of shoots, the explants were inoculated in medium MS, testing the cytokinins 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ) at different concentrations (0.0, 5.0, 10.0, 15.0 and 20.0 μM). Subculture was carried out at 30 days, and the evaluations at 30 and 60 days after inoculation.

For elongation, the plantlets regenerated from treatments with BAP, TDZ and their concentrations were selected and transferred into medium MS with 1 μM gibberellic acid (GA₃), being evaluated after 30 days (time point of 90 days). For rooting, the plantlets arising from treatments with 5 μM BAP and 5 μM TDZ were transferred into medium MS containing NAA (naphthalene acetic acid) or IAA (3-indolacetic acid), at the concentrations of 0.0, 0.25 and 0.50 μM . The evaluations were performed after 30 days in the described rooting media (time point of 120 days).

The media were supplemented with 30 g L⁻¹ sucrose, 0.5 mg L⁻¹ silver nitrate (AgNO₃) (Li et al., 2010) and 7 g L⁻¹ agar. After adjustment of the pH to 5.8, the media were poured into test tubes of 25x150 mm (10 mL) and autoclaved under 1.1 atm at 121°C, for 20 min. All cultures were incubated in growth room with photoperiod of 16/8 h (light/dark), under fluorescent lamps providing flows of 25.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photons, and temperature of 25±1 °C.

Acclimatization of regenerated plants

The plants with approximately 11 cm and with well-developed air part and root, obtained through the rooting treatments, were used for acclimatization. Still in the growth room, at intervals of seven days, the following procedures were carried out: (1) the test tube lids were substituted by film of polyvinyl chloride (PVC); (2) perforations were made in the PVC film; and (3) the film was removed completely. After this period of 21 days, the plants were removed from the tubes, the roots were washed under running water to remove excess culture medium, then transferred individually to matt plastic beakers containing commercial substrate (Vivato Slim®) or corrected dystrophic red-yellow latosol, both autoclaved. At this stage, the plants were nourished with solution of macro- and micronutrients in medium MS, and maintained in a place with light and room temperature.

Morphological evaluations

The phytotechnical evaluations were performed at different phases of the micropropagation, at the times of 30 and 60 days (shoot induction), 90 days (elongation) and 120 days (rooting), as to the number of shoots per explant (NSE); mean length of the shoots (MLS), using a digital pachymeter; percentage of rooting (% Root.); and percentage of survival (% Surv.).

Experimental design and statistical analysis

The experiment followed a completely randomized design (CRD) and each phase (induction, elongation and rooting) was performed with five repetitions per treatment, each consisting of 10 test tubes with one explant each.

In the induction of shoots, the effect of the interaction between the cytokinins and their concentrations was determined in a 3x5 factorial scheme, having three types (BAP, KIN and TDZ) and five concentrations (0, 5, 10, 15 and 20 µM) in the factor cytokinin.

In the elongation phase with 1 µM GA₃, the factorial scheme 2x5 was employed (cytokinin x concentrations), using plantlets obtained through treatments with BAP, TDZ and their different concentrations.

Rooting was conducted in a factorial scheme 2x5, with two cytokinins (5 µM BAP and 5 µM TDZ) and five treatments for rooting: control (0.0 µM), 0.25 and 0.50 µM NAA, and 0.25 and 0.50 µM IAA. The data on rooting percentage (% Root.) was transformed by [(x/100)+1].

The obtained data was subjected to analysis of variance, after verification of the normality and homogeneity. When significance was detected in the interactions or in the factors, the Tukey test of averages was applied on the qualitative factors, adopting 1% and 5% probability, by the software Assisat (Silva and Azevedo, 2009). For the quantitative factor (concentrations), regression analysis was effected with the software Exel (2010), with the factor being represented only with basis on the significance of the coefficients of regression and determination (R²), with levels up to 5% probability being adopted, using the F-test, and taking into consideration the biological phenomenon in study.

Conclusion

A satisfactory frequency of shoot regeneration from apical segments of crambe occurs in media containing 5 µM BAP. Elongation was adequate with 1 µM GA₃. This protocol for plant regeneration *in vitro* may be useful in the process of creation and development of new cultivars in a shorter time using apical explants. There is a need to establish efficient rooting and acclimatization for complete *in vitro* propagation of crambe.

Acknowledgments

Research supported by *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES).

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