In vitro regeneration and acclimatization of plants of Turmeric (Curcuma longa L.) in a hydroponic system

Elsa Ventura Zapata, Guadalupe Salcedo Morales, Ana N Hernández Lauzardo, Blanca Martínez Bonfil, Gabriela Trejo Tapia, Antonia De Jesús Sánchez, Miguel Velázquez Del Valle, ✍ Antonio Jiménez Aparicio

Departamento de Biotecnología. Centro de Desarrollo de Productos Bióticos. Instituto Politecnico Nacional. Km 8.5 Carretera Yautepec-Jojutla. Col. San Isidro, Yautepec, Apdo. Postal 24, Morelos, CP 62731 México. Teléfono:+5201 (735) 394 20 20; Fax: +5201 (735) 394 18 96; E-mail: aaparici@ipn.mx

ABSTRACT

A methodology for the *in vitro* propagation of *Curcuma longa* through indirect organogenesis was developed. The vegetative shoots of the rhizomes grown aseptically in a MS culture medium were used to obtain the plantlets. The dedifferentiation and formation of calluses was achieved using explants of the basal part of the stem of the plantlets developed *in vitro* in the MS medium supplemented with 2,4-dichlorophenoxyacetic (1.5 mg L^{-1}) and Benzyl aminopurine (0.2 mg L^{-1}). The combination of indole-3-acetic acid and Kinetin, promoted the induction of shoot tips and roots and the formation of new plants, which made the transfer of the cultures to another culture medium unnecessary to induce organogenesis. For the *ex vitro* acclimatization of the plants, a hydroponic system was used. This system allowed the adaptation of plants to the gradual decrease of the relative humidity conditions. The plants had a high percentage of survival and continuous growth for six weeks, in which the plants were developed completely.

Keywords: callus, dedifferentiation, organogenesis, propagation, plant growth regulators

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RESUMEN

Regeneración *in vitro* y aclimatación de plantas de Cúrcuma *(Curcuma longa L.)* en un sistema hidropónico. Se desarrolló una metodología para la propagación *in vitro* de *Curcuma longa* vía organogénesis indirecta. Para la obtención de plántulas, se utilizaron las yemas vegetativas de los rizomas crecidos asépticamente en un medio MS. La desdiferenciación del tejido e inducción a callo se obtuvo con explantes de la parte basal de plántulas *in vitro* en un medio de cultivo MS suplementado con ácido 2,4-diclorofenoxiacético (1,5 mg L^{-1}) y Bencil aminopurina (0,2 mg L^{-1}). La combinación de ácido indol acético y cinetina promovió la inducción de brotes y raíces y la formación de nuevas plantas, lo que hizo innecesaria la transferencia de los cultivos a otro medio para inducir la organogénesis. Para la aclimatación *ex vitro* de las plantas se utilizó un sistema hidropónico, el cual permitió la disminución gradual de la humedad relativa ambiental. Las plantas aclimatadas presentaron un alto porcentaje de sobrevivencia así como un crecimiento continuo durantre seis semanas, en las cuales se desarrollaron completamente.

Palabras claves: callos, desdiferenciación, organogénesis, propagación, reguladores del crecimiento vegetal

Introduction

The acceptability of color in a given food is influenced by factors that include its natural pigments as well as the synthetic colorants added. Currently, the preference for natural pigments has increased because of a strong association with their possible positive effects to human health. In this regard, the toxic effects of the synthetic products have also been considered [1, 2]. The carotenoids are a group of mainly lipid-soluble compounds obtained from plants. These pigments contained in natural extracts from saffron, extracted from the stigmas of *Crocus sativus*, paprika from *Capsicum annum* and curcumin of *Curcuma longa*, among others, have been used as food colorants [2]. Curcuminoids are a group of orange to yellow diarylhepatnoids synthesized in the rhizome of Turmeric [3]. Commercial preparations obtained from rhizome extracts contain three constituents with curcumin as the main (75%) fraction as well as two other isomers, demethoxycurcumin and bisdemetoxycurcumin [3]. Turmeric is a tropical plant belonging to the Zingiberacea family. In America, *C. longa* is cultivated in an extensive form in Peru; in Mexico, it was introduced for ornamental purposes and it is known as “Camotillo” or “Raicilla” [4]. The curcumin is widely used as a coloring agent in the food industry where it imparts color to sauces, mustards, soups, dairy and meat products [2, 5], in the pharmaceutical industry because of its therapeutic properties [6], and in the cosmetic industry to brighten and clean the skin. Also, curcumin is used to dye wool and thread in the textile industry [5, 7]. To date, much information exists on the chemical composition of the pigment and its essential oils, as well as its therapeutic properties [6, 8]. In general, turmeric is propagated through underground rhizomes. Since rhizome multiplication in the field is slow, turmeric usually has a high susceptibility to diseases caused by *Phytium myriotylum*, *P. aphanider* and *Pseudomonas solanaraceum* [9]. Also, the rhizome is invaded by nematodes, such as *Exigua ornithogalli* and *Trichobaris trinodata*. The larvae of these insects are developed inside the rhizome, avoiding the proper growth of the plants [10]. These problems do not permit the vegetative reproduction

✍ Corresponding author

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of turmeric, they reduce the yield and the quality of the product, causing the death of the plant including economically important losses [11, 12]. Therefore, a rapid multiplication method is required to provide enough disease-free plant material. The *in vitro* micropropagation and regeneration of plants is a biotechnological alternative for which the intensive culture of phytopathogen-free plants is possible in less time compared to field production [13]. Also, special attention must be given to the *ex vitro* conditions in a green house to allow for the successful development of the micropropagated plants [9]. *In vitro* propagation and regeneration of plants belonging to the Zingiberaceae family has already been reported, i.e. *Alpinia galanga* and *Zingiber officinale* [14]. Nevertheless, turmeric has not received much attention since only a few reports of this important crop are available. In the present paper we report the regeneration of turmeric from shoot tips, the effect of different growth regulators and the *ex vitro* acclimatization of the plants obtained using a hydroponic system which promotes a good plant survival when these are transferred to the field.

Materials and Methods

Plant material: Rhizomes of turmeric *(C. longa* L.) var. "Salem" were obtained in a local market for agricultural products in Mexico City. The botanical classification of the plant was confirmed by the Department of Botany of the National School of Biological Sciences (ENCB-IPN) of Mexico.

Disinfection of the plant material. The rhizomes were surface-sterilized in a water/soap solution for 20 min., followed by rinsing 4 times with sterile distilled water. Under aseptic conditions the rhizomes were disinfected with a solution of mercurial chloride (0.07%, w/v) and Tween 80 detergent (0.05%, v/v) for 10 min., with constant stirring, followed by rinsing 5 times with sterile distilled water. Afterwards, the disinfected rhizomes were surface-dried at room temperature under sterile conditions in a hood with airflow for 15 min.

Plantlet production. About 50 vegetative shoot tips (0.5–1 cm) were separated from the disinfected rhizomes and plated on 35 mL of an agar-solidified (8 g L^{-1}) Murashige and Skoog [15] medium (MS medium) contained in 375 mL "Magenta"-type vessels. The MS medium was supplemented with 30 g L^{-1} of sucrose, and with the vegetal growth regulators reported by Nadagauda *et al.* [13]: 6-benzyl aminopurine (BAP, 0.2 mg L^{-1}) and (α-napthaleneacetic acid (NAA, 0.1 mg L^{-1}). Afterwards, the pH was adjusted to 5.8 and the medium was sterilized by autoclaving at 121 °C (equivalent to 1 Kg cm^{-2} of pressure) for 20 min. The MS medium, the sucrose and the vegetable growth regulators were obtained from Sigma (Saint Louis, Missouri, USA) and the agar was from Bioxon (Mexico). The shoots were incubated at 25 ± 2 ºC, under daylight fluorescent illumination (200 µ mol m^{-2} s^{-1}) with a 16 h photoperiod. The contaminated-cultures were eliminated periodically and the aseptic plantlets were obtained after two months. To prevent the overexposure to plant growth regulators, the plantlets were transferred to a MS medium without the regulators after the first month of growth. These plantlets were subsequently used to obtain aseptic explants for callus induction.

Selection of explants and the effect of plant growth regulators (PGR) on callus induction. In a first stage, it was necessary to select the explants type and the auxin/cytokinin combinations that had the best response for the dedifferentiation process. Different explants from the leaf, shoot, root and basal region of the shoot of the aseptic plantlets were plated in the MS medium with 30 g L^{-1} of sucrose and 8 g L^{-1} of agar added with the auxins 2,4 diclorophenoxiacetic acid (2,4-D), 3-indol acetic acid (IAA) and NAA in a concentration of 1 mg L^{-1} and the cytokinins kinetin (KIN) and BAP in a concentration of 0.1 mg L^{-1}. A medium free from PGR was used as a control. In each treatment, a Magenta vessel containing ten explants was considered as an experimental unit; the number of repetitions was six (n=6). After six weeks, the percentage of callus induction (% IC) was evaluated according to the following relation:

% IC = (explants forming calluses/total number of explants) x 100.

In a second experiment the effect of the concentration of the auxin and cytokinin on callus induction was tested. The explants selected in the first experiment, were plated in the MS medium with 30 g L^{-1} of sucrose, 8 g L^{-1} of agar and the selected auxin – cytokinin combination. The PGR were added to the medium according to an experimental design with the following factors and levels: a) auxin, in a concentration of 0.5, 1.5 and 2 mg L^{-1} and b) cytokinin, in a concentration of 0.05, 0.15 and 0.2 mg L^{-1}. A medium-containing 1.0 and 0.1 mg L^{-1} of auxin and cytokinin respectively was used as a control. In each treatment, a Magenta vessel containing ten explants was considered as an experimental unit; the number of repetitions was six (n=6). After 41 days, the percentage of callus induction (% IC) was determined according to the above-mentioned procedure; additionally, certain characteristics of the calluses (brightness, friability and necrosis or oxidation) were observed through stereoscopic microscopy (Leica) according to their visual appearance. In both experiments, the incubation conditions were: light 200 µ mol m^{-2} s^{-1}, photoperiod of 16 h and temperature of 21 ± 1.5 ºC.

Induction of shoots and regeneration of plants: The following step consisted of promoting the redifferentiation process to obtain new plants. The effect of different auxins and cytokinins on the induction of shoots and their transformation to plants was evaluated using the best callus tissues produced. The conversion of shoots to plants was determined by root formation. A small portion (0.5 mm) of the callus was plated in the MS medium with 30 g L^{-1} of sucrose and 8 g L^{-1} of agar. The combinations of PGR were added according to an experimental design with the following factors a) Auxins 2,4-D, IAA and NAA and b) cytokinins KIN and BAP; both kinds of PGR were added according to the selected concentration obtained in the previous experiment. A medium without growth regulators was used as a control. In this case the incubation conditions were: light 200 µ mol m^{-2} s^{-1}, a photoperiod of 16 h and a temperature of 21 ± 1.5 ºC. In each treatment, a Magenta vessel containing five calluses was considered as the experimental unit; the

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number of repetitions was six (n=6). After 4 weeks, the following parameters were determined: a) the ratio between shoots and calluses and b) the number of plants per callus.

Acclimatization: After the plants were obtained by the redifferentiation and regeneration of calluses under *in vitro* conditions, the following step consisted in the acclimatization of plants. First, the plants were extracted from the Magenta vessels; the leaves were sprinkled with distilled water and the excess agar in the roots, was carefully eliminated. Afterwards, the roots were rinsed using sterile water and the nutritive solution of Hoagland and Arnon [16]; to prevent a possible contamination during the acclimatization, the roots were placed in a solution of "Benlate" (0.2% v/v) for 10 min. The plants were acclimatized in the hydroponic system (Figure 1), developed by Ventura [17]. Basically, the system consists of small cups (8 cm diameter x 5 cm height) containing 200 mL of the nutritive solution of Hoagland and Arnon and covered with aluminum foil; the plants were supported in the vessels through 5 mm diameter holes opened in the foil. This device has the advantage that the roots are always in contact with the nutritive solution, allowing an appropriate intake of nutrients. Each vessel was covered with a transparent polyethylene bag (30.5 x 19 cm), creating a micro-environment with a high relative humidity, through which light can pass while protecting the plants against a possible mechanical damage or the attack of insects. Afterwards, the bags were gradually opened, periodically perforating, (each week), small holes (5 mm diameter) in the bag. This process enabled the gradual decrease in relative humidity, until this factor reaches greenhouse conditions; avoiding drastic environmental changes, which can produce stress. Ten hydroponic systems were built and three plants were placed in each one. Changes in the number of leaves and roots as well as relative humidity were followed during the acclimatization process. Relative humidity was measured twice a day using a thermo-hygrometer (Manis, PTH8708) and the weekly data was the average of the daily data recorded. The plants that grew were transferred to pots containing a mixture of sterile soil and agrolite (1:1 w/w). After eight weeks, the total growth of the plants was evaluated and the specific growth speed (cm d^{-1}) was calculated from their kinetic behavior [17]. Finally, survival percentage was evaluated according to the following relation:

Survival (%) = (# of plants showing growth of leaves and roots/ total # of plants) x 100

In order to compare the hydroponic system with a conventional acclimatization system, 30 plants with a length similar to the micropropagated plants, were cultivated under greenhouse conditions. These control plants received the same conditioning treatment of rinsing, elimination of extra agar and aspersion of water on the leaves. Afterwards, they were planted in pots containing a mixture of sterile soil and agrolite (1:1 w/w). The pots were placed on a tray covered with a transparent polyethylene film. After 30 days, 10% of the cover was opened, on the 45th day, 50% of the plastic film was opened and finally, the cover was completely eliminated after 60 days and the survival was evaluated according to the above-mentioned relationship.

In both cases, the conditions for growth were a temperature of 25 ± 2 ºC, a photoperiod of 16 h of light and a luminosity of 250 µ mol m^{-2} s^{-1}.

Statistical analysis. The one-way ANOVA ($p \leq 0.05$) and the multiple comparison procedure of Tukey were applied to the experiments on the effect of the plant growth regulators in the cellular dedifferentiation and the induction of shoots. The growth profile of the plants was adjusted through linear regression [18].

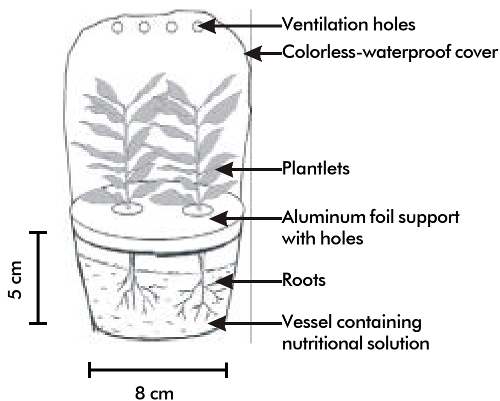

Figure 1. Representative scheme of the hydroponic system used for the acclimatization of Turmeric *(C. longa)* plants.

Results and Discussion

Formation of plantlets. Since vegetative shoot tips were taken from *in vivo* rhizomes, the establishment of contaminant-free culture is a laborious process. The use of mercurial chloride (0.07%, w/v) and Tween 80 detergent (0.05%, v/v) to rinse the explants of the vegetative material drastically reduced contamination. About 90% of the shoots plated in the MS medium with NAA and BAP formed contamination-free plantlets after 2 months. This result agrees with the previous report of Nadgauda *et al*. [13], in which the combination of BAP and NAA promoted the induction of plantlets. Contaminated samples were eliminated and the healthy plantlets were used to obtain explants for the induction of calluses. Also, the development of two or three auxiliary buds obtained from the base of the main shoot was observed after 4 weeks. The buds grew vigorously and formed plantlets (8–10 per Magenta vessel) in the MS medium without plant growth regulators.

Effect of plant growth regulators (PGR) on the induction of calluses. The most vigorous plantlets (Figure 2A) were selected to obtain aseptic explants for cellular dedifferentiation (Figures 2B and 2C) and later transformation and regeneration of new plants (Figure 2D). Table 1 shows, the results of the treatment with different explants and combination of PGR. It was generally observed that the segments from the basal part of the stem showed the best response (45%), with 2,4D and BAP. Also, these calluses showed the best visual aspect, because they were bright, friable and neither necrosis nor oxidation was observed (data not shown). The response using another kind of explant or PGR combinations was significantly lower or no response was obtained. It has been reported

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Figure 2. Photomicrographs showing the cellular dedifferentiation process in C. *longa* L., var. "Salem". A) Plantlets obtained *in vitro* starting from vegetative shoot tips of the rizhome. B) Initiation of the dedifferentiation process induced by the combination of PGR, using explants from de basal region of the plantlets. C) Friable, bright and non-necrotic calluses growing in the basal region of the shoots. D) The redifferentiation process starting from the callus, showing the new and well-developed structures (leaves).

that the induction of the callus is easily obtained from young tissues with meristematic activity or parenkimatose cells. Also the response depends on the chemical nature, the combination and the concentration of PGR and varies according to the plant species and to the origin of the explants [19]. From this, it may be assumed that the response of the base of the stem of *C. longa* was due to the existence of a meristematic area observed in the basal disk (Figure 2B), from which the leaves and the roots were produced, as occurs for example, in the graminae plants. At the same time, here it was necessary to add an auxin and cytokinin to the culture medium to promote cell dedifferentiation and the formation of calluses of *C. longa* (Figure 2C). In contrast, Nadgauda *et al*. [20] induced the formation of calluses using the basal part of the stem and promoted cell dedifferentiation using an induction medium supplemented with KIN (0.1 mg L^{-1}) or BAP (0.2 mg L^{-1}), depending on the age of the explants. These authors conclude that the morphogenic response was mainly due to the presence of the cytokinins. In the present work, the results showed that it was necessary to add both PGR. According to this, the basal region of the stem and the combination of 2,4D and BAP were selected for callus induction.

The following stage consisted of selecting the concentration of PGR to obtain the best response for callus formation; the results are shown in Table 2. It may be observed that the treatment with 2,4-D-BAP in a concentration of 1.5 and 0.2 mg L^{-1} respectively, presented the highest induction (89%) and the best visual characteristics; while in the other concentrations tested, including the control culture, the response was positive, but significantly lower ($p \leq 0.05$). This behaviour has been reported for other species, e.g. *Tagetes minuta* [21] and *Allium cepa* [22]. There is enough evidence to demonstrate that, the addition of an auxin and a cytokinin to the culture medium in a

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Table 1. Response[a] to callus induction (CI, %) using different combinations of PGR[b] and sources of explants of *C. longa*.

PGR combination	Source of explant			
	Leaf	Shoot	Root	Basal region
Control	NR[c]	NR	NR	10
2,4 D/ KIN	NR	5	NR	38
2,4 D/ BAP	5	15	5	45
IAA/ KIN	5	NR	NR	NR
IAA/ BAP	NR	NR	NR	NR
NAA/ KIN	NR	NR	5	10
NAA/ BAP	NR	NR	NR	NR

[a]: The response was evaluated after six weeks.
[b]: Auxins = 1 mg L^{-1}; cytokinins = 0.1 mg L^{-1}
[c]: NR = No response was obtained.

Table 3. Effect of the PGR[a] combination in the formation of shoots and the transformation into plants in *C. longa*.

Combination	Shoots/Callus	a	Plants/Callus	b
Control	1.0 ± 0.2	C[b]	NR	B
2,4D/ KIN	NR	C	NR	B
2,4D/ BAP	NR	C	NR	B
IAA/ BAP	7.0 ± 3.5	AB	4.0 ± 1.52	AB
NAA/ BAP	13.0 ± 2.2	A	3.0 ± 2.24	AB
IAA/ KIN	8.0 ±2.6	AB	7.0 ± 1.67	A
NAA/ KIN	4.0 ± 2.5	BC	3.0 ± 2.19	AB

[a]: The concentration of PGR was selected according to the results obtained in the induction of calluses (see text for explanation); NR = No response was obtained.
[b]: The same letters indicate that there are no significant differences ($p \leq 0.05$) according to the Tukey test.

ratio of 10 to 1 (approximately), promoted callus formation from different explants [23]. The results of the present study confirms the importance of the PGR in the dedifferentiation process of *C. longa* and that its absence inhibited this behavior. According to the results of both experiments, the selected combination of plant growth regulators to induce the formation of callus tissue in the MS medium, was 2,4-D and BAP in a concentration of 1.5 and 0.2 mg L^{-1} respectively.

Induction of shoots and transformation into plants. The following step consisted of promoting indirect organogenesis, with the callus tissue. In this stage, it was necessary to test different combinations of PGR to promote the redifferentiation process, marked by the formation of shoots. The results are presented in Table 3. It was observed that in the control culture (without regulators) the response was low ($p \leq 0.05$) compared to the other treatments, while 2,4-D combined with both cytokinins inhibited the induction process. In contrast, when the combination of NAA and BAP was applied, a higher induction of shoots was obtained ($p \leq 0.05$); in the case of IAA, the response was similar for both cytokinins. These results do not agree with those of other authors [20] in which the induction of shoots in an MS medium has been reported, using a combination of 0.1 mg L^{-1} of KIN and 0.2 mg L^{-1} of BAP and incubating them under light conditions (aprox. 150 μ mol m^{-2} s^{-1}, 12 h photoperiod). In that report, the induction of shoots was achieved precisely by the addition of cytokinins. Additionally, in the present work, the shoots presented a well developed root system, contrary to that reported by other authors, in which the roots could not develop properly [13]. In the same way, it was found that the IAA-KIN treatment showed the best response in the transformation to plants ($p \leq 0.05$), being two-fold higher than that reached by other treatments (Table 3). It was observed that the root system formed with this treatment, presented well-developed and widely branched vigorous roots. On the other hand, the combination of BAP with both auxins did not have an important effect on the formation of roots ($p \leq 0.05$). The positive effect of the auxins, specifically NAA, IBA and IAA in the formation of roots in the shoots is widely reported. On this respect, Nadgauda *et al*. [20], reported the induction of a well developed root system in *C. longa* after transferring the shoots to a White's fresh liquid medium [24] supplemented with 0.25 mg L^{-1} of sodium molybdate and copper chloride, 10% vitamins and without PGR. Also, Sharma and Singh [25], when cultivating meristems of *Zingiber officinale* Rosc. in an MS medium supplemented with 2 mg L^{-1} of KIN, 2.0 mg1 of NAA and 20 g L^{-1} of sucrose, obtained an average of seven plants per bud, similar to that obtained in this study. Additionally, the combination of auxins and cytokinins in the MS medium proposed in this paper, promoted the formation of shoots, as well as roots, making it unnecessary to transfer the cultures to another medium to induce organogenesis thus reducing cost and the possibility of contamination by excessive handling.

Acclimatization of plants. The results corresponding to the increase in the number of leaves and roots during the acclimatization process are shown in Figure 3. In general, it was found that both, the number of leaves and roots in the hydroponic system increased, doubling the original number of these new structures after eight weeks. On the other hand, in the control plants the increase in the number of both structures was small and non significant ($p \leq 0.05$). The relative humidity in the hydroponic system presented a constant decrease, allowing the gradual acclimatization of the regenerated plants to the new environment. It was observed that the formation of new roots occurred between days 20th and 30th after the plants were placed in the hydroponic system, in contrast to the control in which the development was observed at the end of the acclimatization process, after the 40th day. The roots had a vigorous aspect (Figure 4) and readily ramified. It is clear that the development of a vigorous

Table 2. Effect of the different concentrations of 2,4 D and BAP in the callus response of *C. longa*.

2,4 D	BAP	C.I[a]	B[b]	F	N
Control	Control	8 C[d]	(+)	(+)	NR
0.5	0.05	NR[c]	NR	NR	(+++)
0.5	0.15	5 C	(+)	NR	(+)
0.5	0.2	5 C	(+)	NR	NR
1.5	0.05	31 B	(++)	(+)	NR
1.5	0.15	45 B	(+)	(++)	(+)
1.5	0.2	89 A	(+++)	(+++)	NR
2.0	0.05	5 C	NR	(+)	(++)
2.0	0.15	NR	NR	NR	(++)
2.0	0.2	NR	NR	NR	(+++)

[a]: C.I = Callus induction (%), evaluated after 4 weeks.
[b]: B = Brightness, C = Friability, N = Necrosis.
[c]: NR = No response was obtained, (+) favorable response
[d]: The same letters indicate that there are no significant differences ($p \leq 0.05$) according to the Tukey test.

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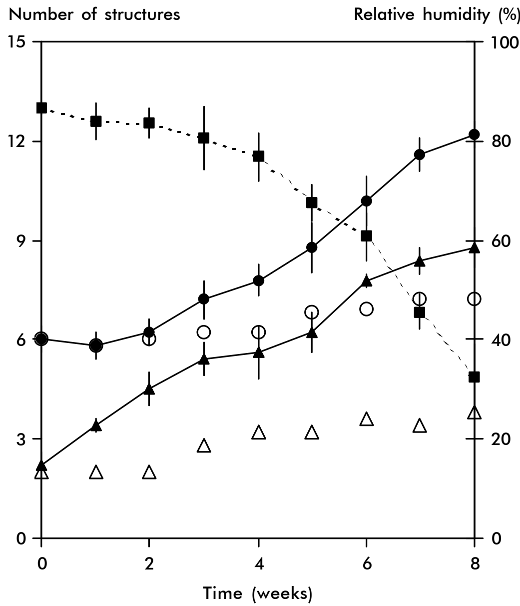

Figure 3. Development of new structures in the plantlets of turmeric during the acclimatization process in the hydroponic (HS) and conventional (CS) systems: roots (—●— HS, —○— CS) and leaves (—▲— HS, —△— CS) contrasted with the gradual decrease in relative humidity (--■--).

radical system in the regenerated plants is a critical factor for a successful acclimatization as well as to obtain a significant increase in the number of rhizomes, an important commercial quality characteristic of the new plants [9, 13, 14]. In this respect Zobayed *et al*. [26], when comparing the *in vitro* growth and development of plants of *Ipomoea batata* cultivated on five different support materials, found that the highest percentage in survival after acclimatization, was attributable to an appropriate development of the root system. Also, the changes in relative humidity significantly affect some of the events that occur during the acclimatization of plants in the greenhouse, as the regulation of the stomatal opening and closing, the biosynthesis of the epicuticular wax and the development of the internal structure of the leaf [27-29]. Indeed, the roots of the *in vitro* plants are fragile and susceptible to mechanical damage. For this reason, the plants can die a few days after they are transplanted in the pots [26, 30]. Since the methodology used in this work is based on the gradual decrease of relative humidity, it is probable that these modifications in the environmental conditions induced the events related with the changes in the structure and function of the leaves; additionally, the hydroponic system, provides the necessary nutriments for plant growth [17]. These conditions are favorable for the invigoration of the *in vitro* roots as well as the absorption of minerals for the autotrophic-growth of *C. longa* plants, since the solution does not contain any carbon source. Nevertheless, these assumptions must be confirmed by experimentation.

Figure 4. Photograph showing the radical system of the *C. longa* plantlets developed *in vitro* and acclimatized in the hydroponic system. It is possible to observe many well developed and ramified roots.

Figure 5 shows the results of the development of the aerial parts of plants acclimatized in the hydroponics and the conventional systems (control), and transferred to pots. It is possible to observe that after six weeks the best growth as well as the maximum survival percentage were obtained with the plants acclimatized in the hydroponic system. In this case, the specific growth speed was 9.7×10^{-1} cm d^{-1} against 3.6×10^{-1} cm d^{-1} in the control group. Also, the percentage of survival of the acclimatized plants using this technology was 90%, whereas the plants in the conventional system suffered a drastic decrease. This behavior could be the result of two factors: a) the exposure of the aerial part to a gradual reduction in relative humidity, thus decreasing stress and b) the vigorous and well developed root-system of

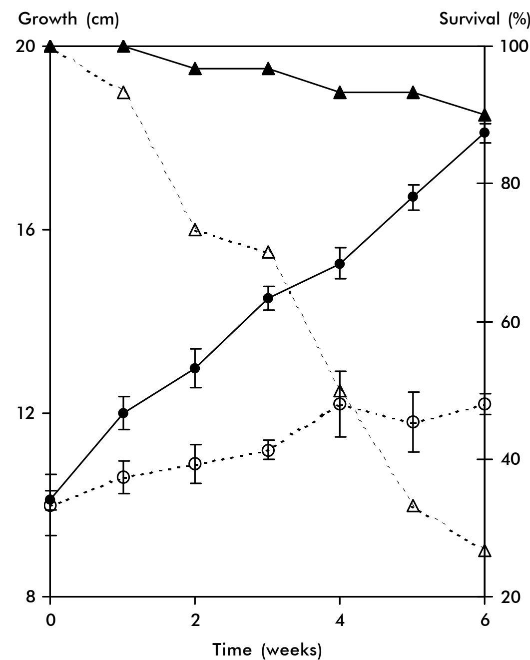

Figure 5. Growth and survival of plants of *C. longa* developed *in vitro* and acclimatized in the hydroponic system (HS), compared with a conventional system (CS): mean of the growth of the aerial part of the plant (—●— HS, —○— CS) and survival of the plants six weeks after their transfer to pots (—▲— HS, —△— CS).

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the new plants that allow the adequate nutrient intake. Both factors helped adapt the plants placed in soil under greenhouse conditions. Similarly, the results of a previous study had shown that the growth of the aerial part and the high percentages of acclimatized plants of *Medicago sativa* L. var TO-70-34 were obtained with those factors [17].

This data, based on the behavior of the plants indicates a striking relationship in the percentage of acclimatization and the development of the root system suggesting the applicability of this method. Furthermore, the hydroponic method described, could reduce losses due to disease or insects in addition to the laborious, uneconomical and time-consuming procedure followed conventionally. Up to now, the acclimatization of *in vitro* cultivated plants, has not been sufficiently developed [30], making it necessary to study some of the physiologic changes of the plants during this process, to achieve a high percentage of survival.

Conclusions

The best callus induction was obtained with the combination of 2,4-D and BAP in a concentration of 1 and 0.2 mg L^{-1} respectively, using the basal part of the stem of *C. longa* plantlets. The transformation into plants was promoted when IAA and KIN were added to the MS medium. This response depended on the combination and concentration of the PGR, which additionally promoted the formation of new shoots, as well as roots, making it unnecessary to transfer the cultures to another medium to induce organogenesis. The hydroponic system proposed, allowed high levels of acclimatization and survival of the *in vitro* plants when these are transferred to pots, probably due to the gradual and controlled decrease of the relative humidity. Because the acclimatization of plants is not so clear, it would be interesting to evaluate the biochemical, physiological and structural changes in the tissues of the leaves during this process.

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