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Micropropagation of clonal lines of thorny artichoke (*Cynara scolymus* L.)

Micropropagación de líneas clonales de alcachofa con espinas (*Cynara scolymus* L.)

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Abstract

The aim of the study was to evaluate the in vitro propagation ability of 10 clonal lines of thorny globe artichoke (*Cynara scolymus* L.). The study methodology comprised five stages of evaluation. The stages evaluated were initiation, multiplication, rooting, acclimatization, and transplant to the field. The study began with the initiation of dissected shoot tips of 10 clonal lines in test tubes containing the Murashige and Skoog (MS) medium. Best results were obtained when explants were cultured on an induction medium containing MS + naphthalene acetic acid (NAA) 1.0 mg l^{-1} + benzyl aminopurine (BA) 1.0 mg l^{-1}, highlighting clonal lines L-250, L-132, and L-62. Because of high rates of vitrification and phenolization in the initial stage, clonal lines L-24, L-127, and L-142 were discarded from the study. Therefore, only seven clonal lines were included for evaluation in the multiplication stage. Once the microplants were obtained under laboratory condition in the culture medium, they were immediately transferred to a proliferation medium containing MS + BA 1.0 mg l^{-1}. Only in three clonal lines (L-132, L-200, and L-250), a high multiplication rate (3.5 shoots/explant) was achieved with axillary bud formation. Of the seven clonal lines evaluated, clonal line L-250 achieved the highest rates in the variables shoot height (3.38 cm), number of leaves (13.4), and number of shoots/explant (4.4). In the rooting stage, clonal line L-250 obtained a significant improvement by transferring plantlets to direct acclimatization after 20 days of in vitro root induction in a medium containing MS + NAA 1.0 mg l^{-1}. Similarly, in the acclimatization stage, the clonal line L-250 showed a significant result. Then, in the transplantation stage, the plants were transplanted to the field with 100% rooting; 30 days after the transplantation, the clonal line L-250 obtained 100% survival in the field than the control treatments (offspring from two locations were used – Mito and Alayo). As the rooting period is reduced by approximately 20 days by inducing direct root formation under greenhouse conditions, the micropropagation technique is optimized with the protocol used in this study.

Keywords: *offspring, clonal propagation, rooting, acclimatization, thorny globe artichoke*

Resumen

El objetivo del estudio fue evaluar la capacidad de propagación *in vitro* de 10 líneas clonales de alcachofa con espinas (*Cynara scolymus* L.). Las fases evaluadas fueron: inicio, multiplicación, enraizamiento, aclimatación y trasplante a campo. El cultivo se inició con la disección de los meristemos de material procedente de las 10 líneas clonales. Los mejores resultados se obtuvieron cuando los explantes se cultivaron en un medio de inducción MS + ANA 1.0 mg l^{-1} + BAP 1.0 mg l^{-1} destacando L-250, L-132 y L-62. Las líneas clonales L-24, L-127, L-142 fueron descartadas por alto porcentaje de vitrificación y fenolización en la fase de inicio. Siete líneas clonales fueron evaluadas en la fase de multiplicación. Las microplantas fueron transferidas a un medio de cultivo MS + BAP 1.0 mg l^{-1}. Se obtuvo una tasa de multiplicación elevada (3.5 brotes/explante) por la formación de brotes laterales solo en tres líneas clonales. La línea clonal L-250 logró los mejores resultados en la variable altura de brote (3.38 cm), número de hojas (13.4) y número de brotes (4.4 brotes/explante). En la fase de enraizamiento, la línea clonal L-250, logró una mejora sustancial en el enraizamiento, al transferir las microplantas después de 20 días de estar en el medio de enraizamiento MS + ANA 1.0 mg.l^{-1} a la aclimatación directa; asimismo, en la fase de aclimatación en campo a los 30 días después del trasplante logró 100% de prendimiento superando a los tratamientos testigos provenientes de esquejes de dos localidades (Mito y Alayo). La técnica de micropropagación se optimiza con el protocolo utilizado, el periodo de enraizamiento se reduce en 20 días aproximadamente al inducir la formación directa de la raíz en condiciones de invernadero

Palabras clave: *Esquejes, propagación clonal, enraizamiento, aclimatación, alcachofa sin espinas*

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Introduction

The artichoke (*Cynara scolymus* L.) is a vegetable native to the Mediterranean basin. It can be reproduced sexually and vegetatively (Jana, 2018). In Peru, the reproduction of thorny artichoke is by vegetative propagation, using offspring and cuttings extracted from mother plants after harvest; and in the case of hybrids of thornless globe artichoke, the propagation is using seedlings (Pariona, 2015). However, in vegetative propagation, the two main factors that limit the expansion and development of the artichoke are the low multiplication rate and the potential for the spread of diseases. These problems can be sorted out using tissue culture techniques (Catacora, Picho, Olivera, & Villantoy, 2001). In the Junín Region, one of the bottlenecks in the production of thorny artichoke is the lack of uniformity of the heads and plant, an aspect that brings negative consequences for the industry and fresh consumption (MINCETUR, 2015). In order to solve this issue, the method of clonal selection complemented with in vitro propagation was used, which generates varieties of high yield and homogeneity (Jana, Gutiérrez, Contreras, & Alfaro 2012). Hence, genetic breeding and selection of thorny artichokes can respond to the demands of the market for consumption in fresh and processed state. However, it is necessary to develop plant production technologies by in vitro culture, establishing micropropagation protocols of thorny artichoke, especially clonal lines that respond with high multiplication rates.

Biotechnological applications of artichoke are used mainly for micropropagation, virus-free production, obtaining haploid plants, induction of genetic variability by mutagenesis, and molecular characterization (Olivera, 2000; Saavedra del Real, 2018; Gallitelli & Mascia, 2015). Artichoke use is still in an initial phase compared with other plant species, and the biotechnological applications are different in different types of artichoke; in particular, the biotechnological applications are in a more advanced stage for early varieties, while only some experimental protocols have been developed for late varieties. Therefore, it is necessary to continue and deepen the use of modern methodologies and extend it to all types of artichokes (Tavazza, Crino, Ancora, & Pagnotta 2015).

Differential responses in the average multiplication rate (number of plants achieved per meristem in 30 days) of artichoke cultivars were obtained experimentally. After in vitro multiplication, varietal compliance should be taken into account. Often, there is variation in the cultivars of the Precocious Mediterranean group. This group is characterized by heteromorphism of leaves (the first leaves are entire leaves and the following leaves are moderately trimmed) (Cointry, López, García, & Firpo, 1999). The lighting and the photoperiod are the most important factors for the in vitro culture of the artichoke (Narender & Kutty, 1994). Plants use only certain parts of the light spectrum for their lighting needs (Gupta, & Jatothu, 2013). The Argentine artichoke cultivar requires the following incubation conditions for growth: 3000 lux with a temperature of 24ºC and a photoperiod of 16 light hours for a period of 40 days in the beginning phase, with some days in the beginning of darkness (Jana et al., 2012).

In a study by El Boullani, Elmoslih, El Finti, El Mousadik and Serghini (2012) on the propagation of artichoke microplants from seedlings produced by seed, the incubation conditions were 25 ± 1ºC with a photoperiod of 16 light hours and a light intensity of 40 µE m^{-2} s^{-1} until the third subculture, while for the following subcultures, the light intensity was decreased to 20 µE m^{-2} s^{-1}.

Vitrification is one of the main problems that occur in the artichoke micropropagation. It is a condition in which the plant shows a wet and translucent appearance, abnormal development of the leaves, and subsequent necrosis (Olivera, 2000). Increasing the agar content or avoiding the use of gelling agents with high gel strength (Debergh, Haarbaoui, & Lemeur, 1981) prevents vitrification in plants.

In the initiation stage, Olivera (2000) managed to regenerate thornless artichoke microplants of 3–4 cm height with 4–5 leaves after 10 weeks from meristems introduced in vitro in the MS medium supplemented with BAP 0.2 ppm and ANA 0.5 ppm. Bedini, Lucchesini, Bertozzi and Graifenberg (2012) in his study used an induction medium containing MS medium (Murashige & Skoog, 1962) with concentrations of ¼ KNO_3 and ¼ NH_4NO_3 added with BA 0.8 mg l^{-1} and IBA 0.2 mg l^{-1} for the initiation stage; initially, the containers were placed in darkness and then in the light for 3 weeks.

In the multiplication stage, Tavazza et al. (2004) used the MS medium supplemented with NH_4NO_3 400 mg l^{-1}, KNO_3 800 mg l^{-1}, and Ca $(NO_3)_2.4H_2O$ 1000 mg l^{-1} and obtained good-quality shoots with leaves of length 5–6 cm and with full leaf edge. The same results were obtained from shoots with sawn leaves, a morphology frequently associated with juvenility and correlated with the late flowering of plants in the field. A good proliferation rate and good quality of shoots were obtained by adding kinetin (KIN) to the culture medium.

Elia, Conversa, Montervino, and Lotti (2007) obtained better multiplication results using the MS medium (Murashige & Skoog, 1962) supplemented with AIA 5.4 µM + 2iP 4.9 µM + KIN 2.3 µM + BAP 0.4 µM with a cytokinin/auxin ratio of 1: 4—obtained 4.5 shoots/explant with an average shoot length of 41.9 mm. In a study by Bedini et al. (2012), the same culture medium was used as in the initial phase by modifying the content of plant regulators adding BAP 0.03 mg l^{-1} and GA3 0.05 mg l^{-1} in 25 ml containers during the first three subcultures obtaining 4.4 shoots per explant in the cv. Empolese.

The subcultures lasted 3 weeks on average, and then, they were placed in containers with 100 ml of culture medium. In the multiplication phase, El Boullani et al.

(2012) used only the macro- and microelements of the MS culture medium supplemented with vitamins B5 (Gamborg, Miller, & Ojima, 1968), sucrose 20 g l^{-1}, adenine sulfate 40 mg l^{-1}, monosodium phosphate 50 mg l^{-1}, kinetin 1 mg l^{-1}, ANA 0.1 mg $l^{-1}$and phytagel 0.3%.

An average of 17 axillary shoots was obtained per explant of the first generation. Olivera (2000) in the multiplication phase used the MS culture medium supplemented with BAP 1.0 ppm and obtained 3–8 shoots per explant within 8–9 multiplication cycles.

In the rooting stage, Tavazza et al. (2004) achieved 90% of shoot rooting after transferring to a medium containing IAA 10 mg l^{-1} and then to a culture medium without growth regulators. Olivera (2000) carried out the rooting stage for thornless artichokes using two stages. In the first part of rooting stage, the MS medium is supplemented with NAA 1.0 ppm in order to induce the formation of root tips in a month, and then, in the second stage, the microplants are transferred to the MS medium supplemented with IBA 1.0 ppm which enhances the proliferation of roots with length >2 cm in a month. In a study by Campanelli, Tagarelli, Mastro, Morone-Fortunato, & Ruta, (2013), it was found that rooting in the MS medium supplemented with AIA 10 mg l^{-1} forms microplants with root system in a single stage. In another study, the MS culture medium supplemented with NAA (0.1–2.0 mg L^{-1}) and also in combination with IAA (2.0 mg L^{-1}) was used (Rossi & Paoli, 1992). However, Kanakis and Demetriou (1993) obtained good rooting results with high concentration of NAA (2.0 mg L^{-1}).

The final success of the in vitro culture depends on the knowledge and experience to transfer and restore the microplants that develop in laboratory conditions to greenhouse environment conditions. This process is called acclimatization or hardening (hardening-off) of the seedlings under significant low relative humidity conditions and higher intensity of light. In many cases, total success is not achieved, even when the acclimation procedure is followed in detail.

Microplants are difficult to acclimate because of two reasons: They are a heterotrophic mode of nutrition and a deficit control of water loss (Kane 2002). A 71% survival of artichoke cv 'Romanesco' in greenhouse conditions at room temperature in the Mediterranean in autumn in September was achieved by Cavallaro, Castiglione, Di Silvestre and Patané (2006). The relative humidity was reduced gradually during a month by acclimatizing the seedlings in trays of seedbed with commercial substrate of peat moss and perlite (3: 1).

The aim of this research was to develop a micropropagation protocol of thorny artichoke, selecting the clonal line best adapted to the in vitro culture, with a higher rate of propagation and rooting to be included in plantlets productive processes in greenhouse.

Materials and methods

Offshoots extraction and explant disinfection

The project began in 2016 in the main producing areas of the Mantaro Valley. A total of 408 elite thorny artichoke plants were selected for this study. The study was carried out during the months of February and March. Based on previously established parameters, 2040 offshoots were transplanted into Evaluation Plots located in the main thorny artichoke producing area in the town of Huaychulo, district and province of Concepción, Junín Region, Peru. In the Evaluation Plot, vegetative and productive parameters were recorded. Ten outstanding clonal lines of thorny artichoke were selected after evaluation, taking as a base the number of buds per plant in the 408 lines evaluated. This was the main component of the yield per plant.

The research work was initiated in February 2017 in the tissue culture laboratory of the Agriculture Research Station Santa Ana Junin. From the mother plant, offshoots were separated, and they were used on the same day in the laboratory for the dissection of meristem to avoid dying of the vascular tissue.

By washing well and rinsing several times with abundant drinking water, removing all traces of dirt, the soil adhered to the offshoots was removed. A large part of the leaves was eliminated and only the buds were left with two leaves (tap water or drinking water was treated and disinfected with calcium hypochlorite). The explants collected were placed in clean containers. To avoid wilting of the buds as a consequence of the phenolization or necrosis of the meristematic tissue, the explants were processed immediately. The buds were surface sterilized in an aqueous solution of 70% ethanol for 1 minute and then in 2% sodium hypochlorite (commercial bleach) for 10 minutes and subsequently rinsed three times with sterilized water. To avoid phenolization of the explants, treatment with 0.1% ascorbic acid was carried out finally.

Analysis of the data obtained in the stages of in vitro propagation was carried out using the SAS V 8.0 program, performing ANOVA unifactorial variance analysis. A Duncan multiple comparison test was carried out with a confidence level of 95% (the letters symbolize and group, in decreasing order, the average values from letter a onwards according to the similarity between treatments) and statistical differences between groups, to evaluate the significant differences in the ANOVA.

The MS medium, commercial product supplied by Duchefa® (the Netherlands) 4.4 g l^{-1} supplemented with sucrose (sugar) 30 g l^{-1} and also benzyl aminopurine (BA) + naphthalene acetic acid (NAA) was used as the culture medium, or BA + indole acetic acid (IAA) was added. Solidifying agents such as phytagel™ supplied by Sigma® USA, in concentration of 2.5 g l^{-1} and plant agar, commercial product supplied by Duchefa® in concentration of 6 g l^{-1} were used for solidification. The

pH of the culture medium was 5.7. The culture medium was sterilized in autoclave for 20 minutes at a temperature of 121°C and a pressure of 1.2 kg/cm^2.

In the initial stage, the meristems were placed in test tubes with 10 ml of culture medium and kept in darkness for 3–4 days to avoid the phenolization process, and then the illumination was gradually raised to 10–20 µmol m^{-2} s^{-1} until reaching 40–50 µmol m^{-2} s^{-1}. In this stage, 10 elite clonal lines of thorny artichoke were evaluated: 1, L-24; 2, L-60; 3, L-61; 4, L-70; 5, L-80; 6, L-127; 7, L-132; 8, L-142; 9, L-200; and 10, L-250.

The following treatments were evaluated in each clonal line: phytagel was used as the solidifying agent from T1 to T3. The solidifying agent was plant agar from T4 to T6. The components of T1–T6 treatments are as follows:

T1 = MS without growth regulators + Phytagel agar
T2 = MS + ANA 1.0 mg l^{-1} + BA 0.4 mg l^{-1} + Phytagel agar
T3 = MS + IAA 1.0 mg l^{-1}+ BA 0.4 mg l^{-1} + Phytagel agar
T4 = MS without growth regulators + Duchefa agar
T5 = MS + NAA 1.0 mg l^{-1} + BAP 0.4 mg l^{-1} + Duchefa agar
T6 = MS + IAA 1.0 mg l^{-1}+ BA 0.4 mg l^{-1} + Duchefa agar.

The variables evaluated were as follows: length of shoots (cm), number of leaves, callus formation (%), phenolized plants (%), and vitrified plants (%).

In the multiplication stage, when the shoots reach an average length of 2–3 cm, they were transferred from the glass test tubes to Magenta® polypropylene boxes containing multiplication culture medium. The Magenta® containers were stored in the incubation room at 16 hours light and 8 hours dark at 24°C.

Seven clonal lines were evaluated: L-60, L-61, L-70, L-80, L-132, L-200, and L-250.

The treatments evaluated were as follows:

T1 = MS 4.0 g + BA 1.0 mg l^{-1} + Phytagel agar 2.5 g
T2 = MS 4.0 g + BA 1.0 mg l^{-1} + Duchefa agar 6.7 g
The variables evaluated were as follows:
Y1 = Shoot length (cm)
Y2 = Number of leaves
Y3 = Number of shoots.

In the rooting stage, the microplants obtained in the multiplication stage were transferred to the rooting MS culture medium supplemented with growth regulators. Rounded clear polypropylene containers of 500 cc capacity with 60 cc of culture medium were used. Using a scalpel and dissection forceps, all the shoots of the microplants obtained in the multiplication stage were previously separated in the laminar flow chamber. The shoots of length >2 cm were transferred to the rooting medium placing four shoots per container.

The following three treatments were considered for the evaluation:

T1: MS + NAA 0.5 mg l^{-1}
T2: MS + BA 1.0 mg l^{-1}
T3: MS + IBA (indole butyric acid) 1.0 mg l^{-1}.

In the acclimatization stage, the microplants were removed from the culture medium and taken to the acclimatization room and placed in a commercial rooting plant solution composed of auxins (0.1% IBA and 0.4% NAA) for 8 minutes and then transferred to river sand washed and disinfected in a plastic harvesting box covered in the bottom with plastic and with holes for drainage. The acclimatization room was maintained with over 95% relative humidity, and frequent watering of the substrate was carried out. After 5 days, the relative humidity gradually decreased.

In this stage, plantlets rooted in the laboratory (T1) and plantlets rooted in a greenhouse (T2) of the promising clonal line L-250 were evaluated. To carry out the direct rooting in river sand washed and disinfected with steam, the microplants developed in Magenta containers were moved to the greenhouse.

When plantlets developed the root system, they were removed from the sand and placed in a tray with clean water to prevent dehydration. Plantlets were transplanted into plastic bags of 2 L capacity in a peat moss local substrate mixed with imported peat moss in a ratio of 2: 1 for 30 days and then transplanted to the field.

Results

Initial stage

In the initial stage, the two decisive factors for the subsequent multiplication of regenerated artichoke microplants were phenolization and vitrification. In Figure 1, it is observed that L-24, L-127, and L-61 were the clonal lines most affected by phenolization with 41.7%, 33.3%, and 25.0%, respectively.

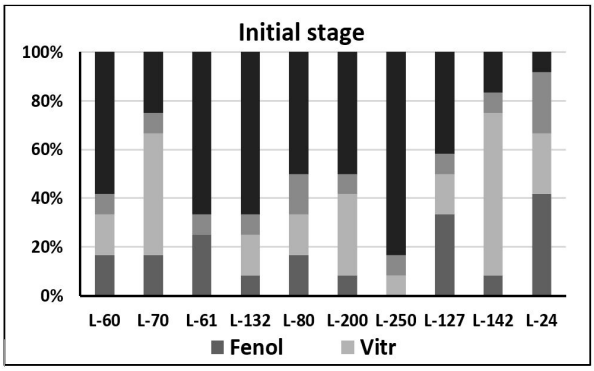


Figure 1. Occurrence of phenolized, vitrified and contaminated microplants of elite clonal lines of thorny artichoke (*Cynara scolymus* L.) in the initial stage

In the case of vitrification, L-142, L-70, and L-200 were the clonal lines with the highest percentage of vitrified microplants with 66.7%, 50.0%, and 33.3%, respectively. In the case contamination, clonal lines with the highest percentage of contaminants were observed in L-24 and L-80 with 25% and 16.7%, respectively; the other clonal lines did not exceed 9% contamination.

In this stage, 10 elite clonal lines were processed and evaluated, which were previously evaluated and selected in the field. After the planting of meristems under aseptic conditions in the laboratory, regenerated microplants were evaluated, highlighting L-80 in T1 and L-61 in T2 with 1.8 cm in height of microplant, as shown in Table 1.

In the T2, in number of leaves, the clonal lines L-127 and L-250 obtained 2.5 leaves/microplant, while in the others the values reached were lower. The treatments were compared based on the solidifying agents.

Multiplication stage

Seven elite clonal lines were processed and evaluated in the multiplication stage. The clonal lines L-24, L-127, and L-142 were discarded due to vitrification and phenolization in this stage (Table 2). The highest shoot height value was for L-250 with 3.38 cm, followed by L-60 with 3.36 cm, and L-132 with 3.32 cm, and no significant differences were found between these treatments. In the variable, number of leaves, the first was L-250 with 13.4 leaves, followed by L-80 with 12.1 leaves, and L-200 with 11.6 leaves. No significant differences were found between these treatments. In the variable, shoot number/explant, the highest result was obtained for L-250 with 4.4 shoots/explant in the first subculture, followed by L-200 with 2.8 shoots/explant, and showed statistically significant differences; whereas no statistically significant differences were observed in L-200 and L-80.

Table 1. Evaluation of morphological parameters of 10 thorny artichoke lines in different treatments of growth regulators and type of agar in the initial stage.

Lines	Shoot height (cm)	No. of leaves	Shoot height (cm)	No. of leaves	Shoot height (cm)	No. of leaves	Shoot height (cm)	No. of leaves	Shoot height (cm)	No. of leaves	Shoot height (cm)	No. of leaves
	T1		T2		T3		T4		T5		T6	
L-60	0.15	0.0	0.50	1.0	0.60	1.0	0.20	1.0	0.75	2.0	0.45	1.0
L-70	0.20	0.0	0.35	1.0	0.45	1.0	0.35	0.5	0.35	1.0	0.15	0.0
L-61	0.85	0.5	1.85	1.0	0.50	0.5	0.40	0.5	1.70	1.0	0.65	1.0
L-132	0.30	0.5	0.85	1.5	1.65	2.0	0.40	1.0	0.70	1.0	0.25	0.5
L-80	1.85	2.0	0.55	1.0	0.70	0.0	0.10	0.5	1.40	2.0	0.65	1.5
L-200	0.45	0.5	1.15	1.0	0.30	1.0	1.30	2.0	0.60	1.5	0.40	1.0
L-250	0.75	1.5	0.65	2.5	0.55	1.5	0.55	1.5	0.85	2.0	0.75	1.5
L-127	0.15	0.0	1.30	2.5	0.25	0.5	0.10	0.5	0.20	0.5	0.20	0.5
L-142	1.20	1.0	1.50	2.0	0.40	1.0	0.30	0.5	0.50	1.5	0.45	1.5
L-24	0.25	0.5	0.30	1.0	0.20	0.5	0.20	1.0	0.30	1.0	0.30	0.5

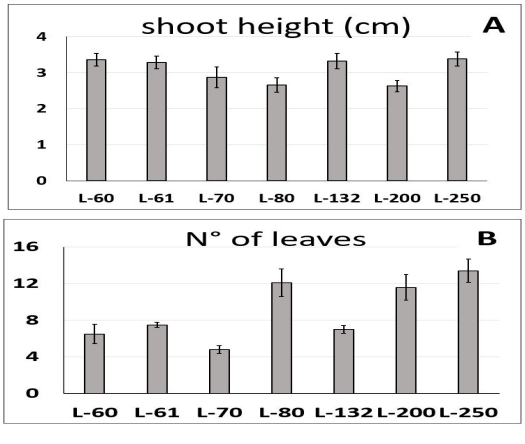
Note: No.: number

Table 2. Morphological parameters for seven thorny artichoke (*Cynara scolymus* L.) clonal lines at the end of the proliferation process.

Y1 = shoot height (cm)		Y2 = number of leaves		Y3 = shoot number/explant	
7 (L-250)	3.38 a	7 (L-250)	13.4 a	7 (L-250)	4.4 a
1 (L-60)	3.36 a	4 (L-80)	12.1 a	6 (L-200)	2.8 b
5 (L-132)	3.32 a	6 (L-200)	11.6 a	4 (L-80)	2.7 b
2 (L-61)	3.28 a b	2 (L-61)	7.5 b	2 (L-61)	2.2 b c
3 (L-70)	2.87 b	5 (L-132)	7.0 b	5 (L-132)	1.7 c d
4 (L-80)	2.66 b	1 (L-60)	6.5 b	3 (L-70)	1.7 c d
6 (L-200)	2.63 b	3 (L-70)	4.8 b	1 (L-60)	1.4 d

Note: Different letters indicate statistically significant differences, according to Duncan's test ($p \leq 0.05$).

In Figure 2A, 2B, 2C, clonal line L-250 exceeded all other lines with 4.4 shoots, 13.4 leaves and 3.38 cm average height, and this was repeated in the following subcultures.



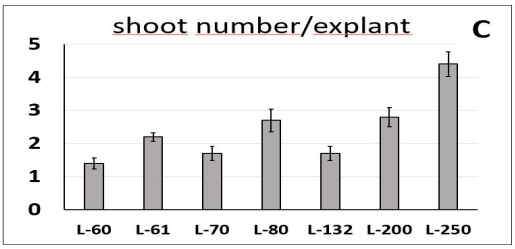


Figure 2. Effect of the genotype of six thorny artichoke lines (*Cynara scolymus* L.) grown on multiplication medium (first subculture): (A) Shoot height (cm), (B) number of leaves, and (C) shoot number/explant. Values are means ± SE (n = 10).

Figure 3 shows the development of explants in clear polypropylene containers and the formation of lateral shoots during the multiplication stage.

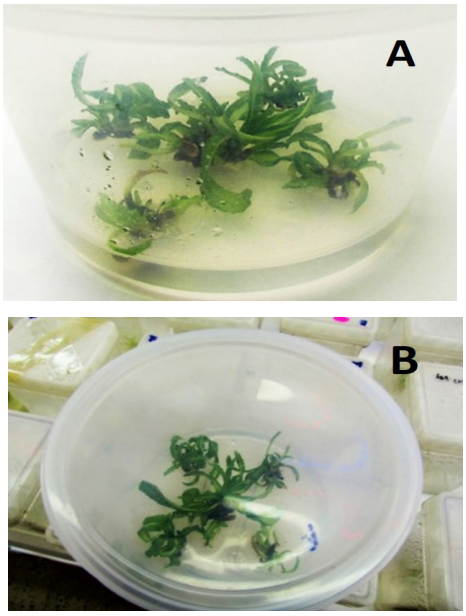


Figure 3. Artichoke microplants in multiplication medium: explants growing in container (A) and formation of lateral shoots (B).

In the second subculture of the three clonal lines evaluated in the multiplication stage, the highest shoot height (3.58 cm), the largest shoot number/explant (5.8), and the largest number of leaves (15.5) were obtained in the clonal line L-250 (Figure 4).

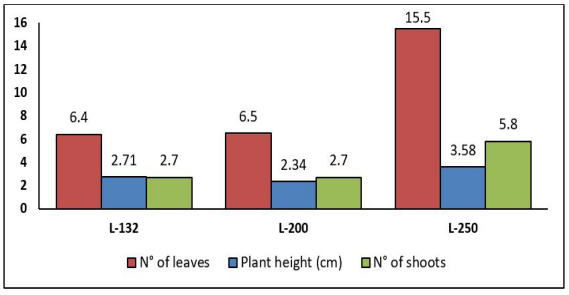


Figure 4. Comparison of morphological parameters of three clonal lines of thorny artichoke (*Cynara scolymus* L.) in multiplication stage.

Rooting stage

In the rooting stage, out of the seven clonal lines only three clonal lines were processed and evaluated, and the other four were discarded, because of vitrification and necrosis.

In the variable "length of shoots" in the treatment with NAA (Figure 5A), the results showed that L-132 obtained a shoot length of 4.1 cm at 45 days, and with a similar evolution, L-250 obtained a shoot length of 4.08 cm, and the response of L-200 was smaller (shoot length 3.84 cm). In Figure 5B where IBA was used, the results highlighted L-250 against L-200 and L-132 with 4.2 cm versus 4.0 cm and 3.7 cm, respectively. In Figure 5C, in the BAP treatment, a greater response was observed in L-250 with 4.3 cm at 45 days compared to the other lines that reached only 3.98 cm.

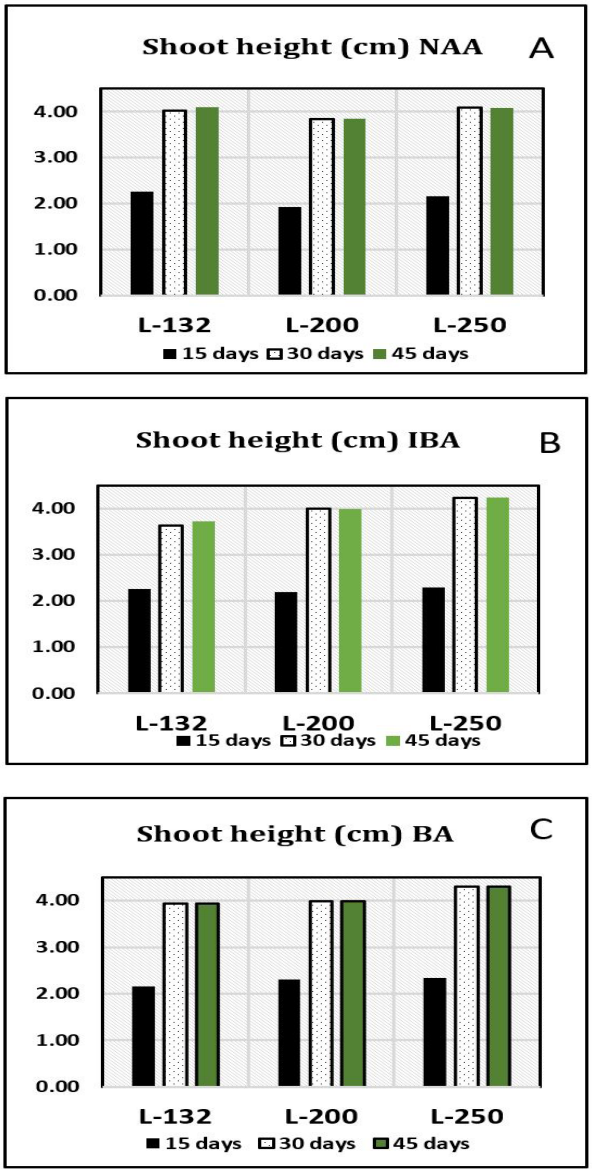


Figure 5. Effect of growth regulators on shoot growing of thorny artichoke microplants (*Cynara scolymus* L.) with different growth regulators during 45 days of rooting stage.

Figure 6A shows that clonal line L-132 had a better response to shoot height, with NAA 1.0 mg l^{-1} (3.90 cm), followed by BA 1.0 mg l^{-1} (3.74 cm), and IBA with 1.0 mg l^{-1} (3.72 cm); however, the best response to root formation of L-132 was 0.54 cm in length of roots with AIB, superior to the treatments with BA and NAA. In contrast, in the number of roots, treatment with BA exceeded the treatments with IBA and NAA. Figure 6B shows that L-200 had a similar shoot length of 4.0 cm with IBA and BA, while it was 3.84 cm with NAA. In root length, a length of 0.68 cm with BA exceeded the treatments with IBA and NAA. Figure 6C highlights L-250 added with BA in the number of roots formed and the length of shoots exceeding 4.0 cm shoot height (T2), 2.2 number of roots (T2), and 0.86 cm root length (T2).

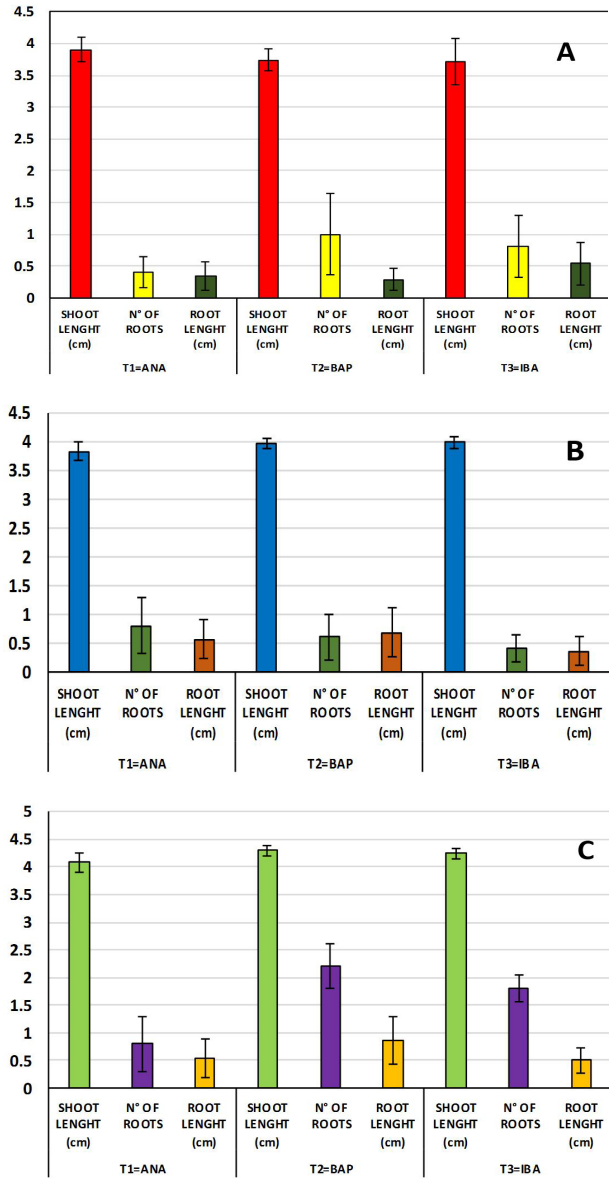


Figure 6. Effect of growth regulators on root development of shoots in three clonal lines of thorny artichoke (*Cynara scolymus* L.) in the rooting stage. (A) L-132, (B) L-200, and (C) L-250. Values are means ± SE (n = 10).

In Table 3, treatment 3 (T3) in L-250 shows a significant difference compared to the other two clonal lines in the shoot length. The clonal line L-250 reached 4.20 cm compared to L-200 and L-132 with 3.92 cm and 3.90 cm, respectively.

Table 3. Shoot height (cm) comparison of thorny artichoke (*Cynara scolymus* L.) of three clonal lines in the rooting stage.

Treatment	Mean	Duncan grouping
3 (L-250)	4.2	a
2 (L-200)	3.92	b
1 (L-132)	3.9	b

Note: Different letters indicate statistically significant differences, according to Duncan's test ($p \leq 0.05$).

Greater phenolization was observed in L-132 and L-200 compared with L-250 in the case of microplants rooted in greenhouse. A greater percentage of difference in the clonal line L-250 was manifested after 30 days of root development (Figure 7).

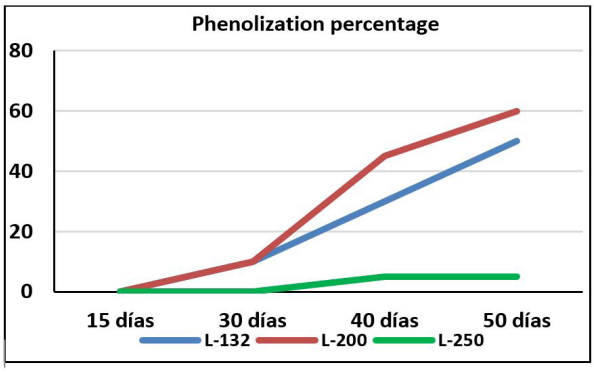


Figure 7. Process of phenolization of thorny artichoke microplants (*Cynara scolymus* L.) of three clonal lines during ex vitro rooting in greenhouse.

Acclimatization

The length of the plantlet on average was 6.0 cm and 7.0 cm for in vitro rooting and ex vitro rooting, respectively, after 15 days of acclimatization (Figure 8A). Because of low multiplication rate, clonal lines L-132 and L-200 were discarded. Figure 8B shows the development of the plantlets after 30 days, reaching an average length of 8.1 cm and 8.8 cm for in vitro and ex vitro rooting, respectively.

The root length and the number of leaves of both the laboratory plantlets and the greenhouse plantlets were, respectively, 15 cm and 15.4 cm, and 4.3 and 4.7 leaves, after 45 days of acclimatization. Both plantlets, from the laboratory and greenhouse, developed a good root system (Figure 8C).

A satisfactory result was obtained with 80% of rooting in a period of 15–20 days with growth of fibrous roots with good root hairs. The rooting stage is shortened in a month and a half with 80% of rooted plants using this method.

The growth of these plants is more vigorous.

The day after the acclimation, the plants were recovered without wilting of leaves, and the plants were again watered, trying not to wet the leaves. The growth of the plants was very high, and hence, they were suitable for transplant in 2 weeks average, when the plants presented very vigorous leaves (4–5 true leaves).

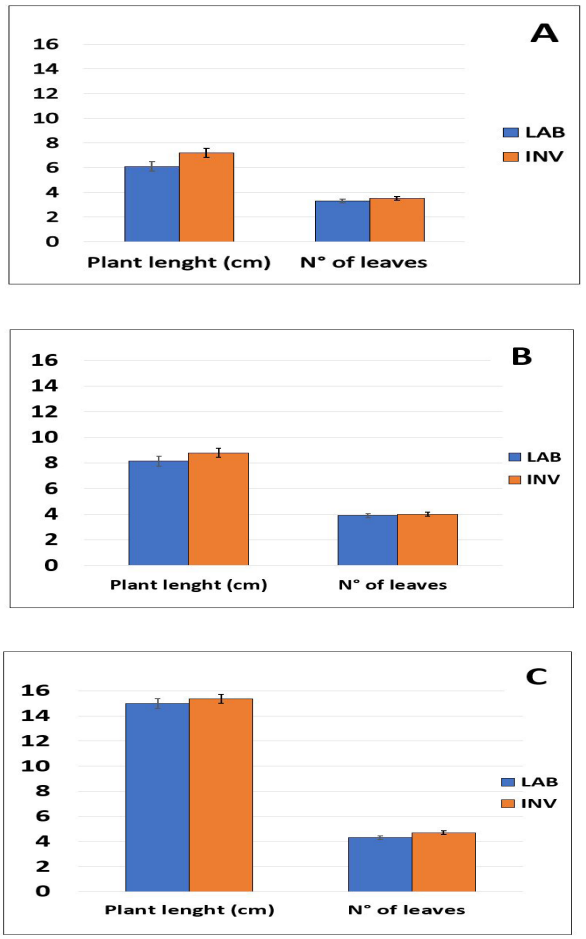


Figure 8. Development of thorny artichoke plantlets (*Cynara scolymus* L.) of clonal line L-250 in greenhouse acclimatization stage. (A) At 15 days, (B) at 30 days, and (C) at 45 days. Values are means ± SE (n = 10).

Transplant and survival rate

The results after the development of the plants in the greenhouse and transplantation to the final field in an Evaluation Trial in two locations (Annex Alayo, district and province of Concepción; and EEA Santa Ana Junín, district of El Tambo, province of Huancayo) and three times of transplant (April, May, and July 2018) are presented in Table 4. At 30 days after the transplant in the field, plants of the clonal line L-250 achieved 100% survival, surpassing the control treatments when used cuttings from locations Mito and Alayo. In addition, these plants did not show rot by bacteriosis in the field, and they showed vigorous development with frost resistance in different phenological stages of the crop.

Table 4. Survival rate of three transplant periods (April, May, and July), in evaluation assay of clonal thorny artichokes, Santa Ana Junín EEA and Alayo Annex, Concepción Province, 2018.

Treatments	Percentage of survival			Mean % of survival by treatment
	First transplant: 26/04/2018	Second transplant: 29/05/2018	Third transplant: 24/07/2018	
T1: L-250	100	100	100	100
T2: Control (offspring – Mito)	90	86.7	100	92.23
T3: Control (offspring – Alayo)	90	98.4	100	96.13

Discussion

In the initial stage, the presence of meristematic tissues with symptoms of phenolization (oxidation and necrosis of the meristematic tissue) may be observed in some cases, and vitrification symptoms (wet and translucent seedlings) may also be observed in some regenerated meristems after days. The two decisive factors for the subsequent multiplication of regenerated artichoke microplants in the initial stage were phenolization and vitrification. In this regard, Morzadec & Hourmant (1997) suggest that phenolization can be prevented by increasing the concentrations of antioxidants in liquid and solid culture media, in obtaining callus and cellular suspensions.

The first subculture clonal line L-250, in the multiplication stage, exceeded all other clonal lines in the variables, such as plant height (3.38 cm), number of leaves (13.4), and shoot number/explant (4.4), at 25 days of cultivation. These results were repeated in the following subcultures. In this way, this line can propagate quickly compared to the others lines. The other lines had the problem of tissue phenolization, besides the disadvantage of the low propagation rate, <2 shoots/explant. The L-250 clonal line showed more number of leaves, which is important for the development of the microplant, while other clonal lines such as L-70 and L-60 showed lesser number of leaves and slower leaf development. El Boullani et al. (2012) in his study used four categories of shoot measurements for subculture, obtaining 7.73 and 7.30 shoots/explant for categories 1–1.5 cm and 1.5–2 cm, respectively. However, only 2.76 shoots/explant were obtained for the >2 category, apparently as a result of apical dominance that inhibits the formation of adventitious shoots; and only 1.06 shoots/explant were obtained for the <1 cm category, of which 26.6% were necrotic, after 2 weeks in cultivation.

Greater phenolization was observed with L-132 and L-200 compared with L-250 in the case of microplants rooted in greenhouse. This difference was manifested in greater dimension in L-250 after 30 days of root development in this phase, because a greater number of microplants were phenolized in these two lines (L-132 and L-200) and ended with a total necrosis (Figure 7).

These microplants left the laboratory with little or no root formation, slightly induced by the auxin ANA. Later in the greenhouse, and on peat moss or sand substrate, they completed the root development in the acclimatization. Larger seedlings developed roots faster than the smaller seedlings, so it is recommended to put seedlings that exceed 6 cm length in sand.

The tolerance to frost in field, of the L-250 line, obtained in vitro, shows the advantage of this material compared to the conventionally propagated plants, because the effect of low temperature did not cause damage to the plants; while in the traditional culture, the frost season is a critical stage for its development (Pariona, 2015) (CIREN CORFO, 1988).

This technique is an alternative to quickly obtain disease-free material, independent of the environmental conditions of production. The only technique that allows the massive multiplication of promising material within breeding programs is the in vitro micropropagation technique (Jana et al., 2012).

Conclusions

In the initial stage, because of a greater tendency to vitrification and phenolization of the tissues, some selected clonal lines present less adaptation to the in vitro culture. Some clonal lines showed low rates of propagation in the multiplication stage, and some have little or no root development in the rooting stage.

The clonal line that is the best adapted to the micropropagation with high multiplication rate and favorable tendency to rapid rooting is the promising clonal line L-250. This clonal line, in addition, has a good adaptation in greenhouse and field, with an optimized protocol to its requirements.

The protocol obtained, based on the micropropagation of the L250 clonal line, made it possible to optimize this technique, because the rooting period is reduced by approximately 20 days by inducing direct root formation under greenhouse conditions.

Acknowledgments

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References

Bedini, L., Lucchesini, M., Bertozzi, F., & Graifenberg, A. (2012). Plant tissue cultures from four Tuscan globe artichoke cultivars. *Central European Journal of Biology, 7*(4), 680–689. DOI: 10.2478/s11535-012-0064-x

Campanelli, A., Tagarelli, A., Mastro, G.D., Morone-Fortunato, I., & Ruta, C. (2013). Innovative large-scale production of early artichoke transplants. *Acta Horticulturae*, *983*, 331–335. DOI: 10.17660/ActaHortic.2013.983.46

Catacora, E., Picho, H., Olivera, J., & Villantoy, A. (2001). Cultivo de alcachofa sin espinas. Serie Manual N° 01 ed. Lima, Perú: INIA. Retrieved from http://repositorio.inia.gob.pe/handle/inia/896

Cavallaro, V., Castiglione, V., Di Silvestre, I., & Patané, C. (2006). *Influence of different transplantation times on the acclimatisation process of micropropagated artichoke*. Murcia, España, ISHS, 10.

CIREN CORFO Centro de información de recursos naturales (1988). *Manual del cultivo de alcachofa*. Publicación CIREN N° 72. Retrieved from bibliotecadigital.ciren.cl/bitstream/handle/123456789/1960/PC07288.pdf

Cointry, E.I., López, F.S., García, S.M., & Firpo, I.T. (1999). Mejoramiento genético del alcaucil (*Cynara scolymus* L.). *Avances en Horticultura, 4*(1), 44–53.

Debergh, P., Haarbaoui, Y. & Lemeur, R. (1981). Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. *Physiologia Plantarum, 53*(2).

El Boullani, R., Elmoslih, A., El Finti, A., El Mousadik, A., & Serghini, M. 2012. Improved in vitro micropropagation of artichoke (*Cynara cardunculus var. scolymus* L.). *European Journal of Scientific Research, 80*(4):430–436.

Elia, A., Conversa, G., Montervino, C. & Lotti, C. (2007). Micropropagation of the Early Artichoke Cultivar Violet du Provence. *Acta Horticulturae, 730*, 127–134. DOI: 10.17660/ActaHortic.2007.730.14

Gallitelli, D. & Mascia, T. (2015). Virosis. In N. Calabrese (Ed.), *La alcachofa y el cardo* (pp. 231–245). Milano, Italia: Euromedia SRL.

Gamborg, O., Miller, R., &. Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research, 50*(1), 151–158. https://doi.org/10.1016/0014-4827(68)90403-5

Gupta, S. D. & Jatothu, B. (2013). Fundamentals and applications of light-emitting diodes (LEDs) in in vitro plant growth and morphogenesis. *Plant Biotechnology Reports, 7*(3), 211–220.

Jana, C. (2018). Germoplasma de alcachofa. In C. Jana & G. Saavedra (Eds.) *Manual de producción de alcachofas* (pp. 23-29). La Serena, Chile: INIA. Retrieved from http://biblioteca.inia.cl/medios/biblioteca/boletines/NR40931.pdf

Jana, C., Gutiérrez, R., Contreras, C. &. Alfaro, V. (2012). Micropropagación de alcachofas: Técnica eficiente para una multiplicación con calidad productiva y sanitaria. *Tierra Adentro, 100*, 28–34. Retrieved from: http://biblioteca.inia.cl/medios/biblioteca/ta/NR38495.pdf

Kanakis, A. & Demetriou, K. (1993). In vitro shoot regeneration of globe artichoke from shoot apices treated with thidiazuron and from mature zygotic embryos treated with cytokinins. *Journal of Horticultural Science, 68*, 439–445.

Kane, M. (2002). Propagation from preexisting meristems. In: R. Trigiano & D. Gray (Eds.) *Plant tissue culture concepts and laboratory exercises*. Second Edition (pp. 75-86) New York: CRC Press.

Ministerio de Comercio Exterior y Turismo (MINCETUR). (2005). *Planes Operativos de Productos Seleccionados*, Junin: Mincetur.

Morzadec, J. & Hourmant, A. (1997). In vitro rooting improvement of globe artichoke (cv. Camus de Bretagne) by GA3. *Scientia Horticulturae, 72*, 59–62.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioessays with tobacco tissue culture. *Physiologia Plantarum, 15*, 473–497.

Narender, S.N. & Kutty, K.K. (1994). Meristem and shoot tip culture: requirements and applications. In I. Vasil & T. Thorpe (Eds.), *Plant Cell and Tissue Culture* (pp. 37-70). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Olivera, S.J. (2000). M*icropropagación de alcachofa sin espinas (Cynara scolymus L.)*. Lima: Serie Folleto Nª 03-00 INIA.

Pariona, M.E. (2015). *Tecnología de manejo del cultivo de alcachofa de exportación en sierra*. Folleto Nº 1 - 15 Ed. Lima, Perú: INIA. Retrieved from http://repositorio.inia.gob.pe/handle/inia/145

Rossi, V. & Paoli, G. (1992). Micropropagation of artichoke (*Cynara scolymus*). *Biotechnology in Agriculture and Forestry, 19*,118–134.

Saavedra Del Real, G. (2018). Clon de alcachofa tipo "Argentina" libre de virus. In C. Jana & G. Saavedra (Eds.), *Manual de producción de alcachofas* (pp. 143–151). La Serena, Chile: INIA.

Tavazza, R., Crino, P., Ancora, G., &. Pagnotta, M. (2015). Il Carciofo e il cardo. Ricerca. In N. Calabrese (Ed.) *Il Carciofo e il cardo*. (pp. 303–313) Milano, Italia: Euromedia S.R.L. Retrieved from: https://www.colturaecultura.it/capitolo/biotecnologie

Tavazza, R., Papacchioli, V. & Ancora, G. (2004). An improved medium for in vitro propagation of globe artichoke (*Cynara scolymus* L.) Acta Horticulturae 660:91-97. DOI: 10.17660/ActaHortic.2004.660.10