In vitro culture *Cynara cardunculus* var. *sylvestris* – callogenesis induction and micropropagation

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Abstract

***Cynara cardunculus* L., a cross-pollinated species complex, has a high level of heterozygosity, giving rise to different phenotypes within populations when propagated by seed. Traditional vegetative propagation of cardoon by offshoot involves the destruction of the mother plant, dissemination of phytosanitary problems, and a low multiplication rate due to the limited number of offshoots produced by the mother plant. Micropropagation is a viable alternative method for large-scale production of selected disease-free plants. The main goal of this work was the study of two in vitro propagation methodologies, micropropagation by shoot tips and callogenesis induction, aiming to multiply disease-free plants of selected Portuguese genotypes. Leaves and offshoots of different genotypes were submitted to two disinfection protocols. For micropropagation from field offshoots, the MS medium combined with IBA and BAP was used, while for callogenesis from leaves, seven combinations of growth regulators (NAA, BAP, IBA, KIN) were studied. The disinfection procedure was important to minimize the contamination rates during the culture establishment phase by shoot tips. The explants overcoming this phase were then multiplicated and rooted. For callogenesis induction, a new protocol of cardoon genotypes was developed for further indirect organogenesis and secondary metabolites production. A higher callus formation was obtained in BAP:NAA (1:10 mg L^{-1}) or BAP:NAA (1:2 mg L^{-1}) media. The type of leaves (young versus adult) seems to be determinant in callus initiation, considering a lower level of contamination obtained with young leaves. For callogenesis and micropropagation by shoot tips, the genotype type influenced the callus formation and induction stage of shoots, respectively. This study represents the first investigation into the in vitro propagation of Portuguese cardoon genotypes. Further studies are being conducted to increase the induction and rooting rates to implement this new knowledge in future cardoon breeding programs.**

Keywords: cardoon, meristems, organogenesis, disease-free plants

INTRODUCTION

Cynara cardunculus L., a perennial herbaceous plant from the *Asteraceae* (*Compositae*) family, is native to the Mediterranean region. This species comprises three varieties: artichoke (var. *scolymus* (L.) Fiori), cultivated cardoon (var. *altilis* DC), and wild cardoon (var. *sylvestris* (Lamk) Fiori) (Folgado and Abranches, 2019). Although the flower of the wild cardoon has traditionally been used in cheese making, recent studies have shown its potential for biomass production, animal feed, biodiesel production, and extraction of bioactive compounds (Ramos et al., 2019; Scavo et al., 2020).

Seed sowing is the most commonly used method for establishing the culture due to its lower cost and the plant's poor tolerance to transplantation (Quintero, 1986). However, the

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high levels of heterozygosity in *C. cardunculus* resulting from its diploid genome (2*n*=34 chromosomes) and cross-pollination can lead to significant variation within seed-propagated populations (Castro et al., 2021). The traditional vegetative propagation has been used widely in the multiplication of artichoke (*C. cardunculus* var. *scolymus*). However, this tool presents some disadvantages. The rate multiplication is low, it leads to the destruction of the mother plant, and the propagules may spread some pathogenic agents, carrying out some phytosanitary problems to the region of the plantation. New tools are needed to overcome these bottlenecks.

Micropropagation techniques, such as meristem culture and callogenesis, have been developed to facilitate the rapid and efficient propagation of artichokes (Bekheet et al., 2014; Ozsan and Onus, 2020b). Meristem culture involves the cultivation of meristematic tissue to produce whole plants. At the same time, callogenesis is the induction of callus formation from plant tissue explants, which can subsequently be regenerated into plants, also known as indirect organogenesis. These techniques offer several advantages over traditional propagation methods, including the production of large quantities of uniform, disease-free plant material in a relatively short period and the ability to produce plants with desirable traits, such as increased resistance to biotic and abiotic stresses and improved quality and yield (Abdalla et al., 2022). In addition, the application of callus induction can be a fast and easy protocol to obtain an abundance of raw material for secondary metabolites production in aseptic conditions without external environmental fluctuations (Meneghini et al., 2016; Pandino et al., 2017a; Joshaghani et al., 2014; Abbas et al., 2021).

Some studies have described the micropropagation of artichoke cultivars propagation (Pandino et al., 2017b; Ozsan and Onus, 2020a, b; Ancona et al., 2021). To our knowledge, no in vitro protocol from adult genotypes of wild cardoon has been described. Therefore, the main objective of this study was to develop a protocol to propagate selected Portuguese cardoon genotypes by multiplication of shoot tips (1), and a protocol for induction of callogenesis (2).

MATERIALS AND METHODS

Plant material

Leaves and offshoots of adult plants from *C. cardunculus* (var. *sylvestris*) were collected from the field located in Beja, Portugal, during three different periods (November 2021, January and May 2022). Three genotypes with added value due to their high production of biomass (G7 and G8) and secondary compounds, namely cynaropicrin (G8 and G9) were selected according to Paulino et al. (2021, 2023) and Belo et al. (2023) and used in this study. The collected samples were stored at 4°C until the in vitro experiments.

Callogenesis

Leaves from the inner part of the plant collected in November 2021 and January 2021 (young leaves), and May 2022 (adult leaves) were used for callus induction. Two different disinfection procedures were applied. The first one was applied in the November collection, following the methodology described by Ozsan and Onus (2020b) (denominated in this work as Protocol DPa). The second protocol (DPb) was applied to plant material from the January and May collections. The plant material in protocol DPb was surface sterilized by washing in tap water with some drops of detergent for 10 min, followed by immersion in 70% (v/v) ethanol for 30 s, immersion in a solution of fungicide (Derosal 1 g L^{-1}) for 20 min, immersion in 70% (v/v) ethanol for 1 min, immersion in a 70% commercial bleach solution with 5% active chlorine for 10 min. Finally, the plant material was rinsed three times with sterile water, in a laminar flow chamber.

Medium culture composition

To evaluate the effect of the type and concentration of plant growth regulators on callus formation, different and ratio of cytokinin and auxin in Gamborg B5 medium were tested using the plant material collected in January, using three genotypes (G7, 8 and 9). The seven culture

media tested are described in Table 1. As cytokinin (6-benzylaminopurine (BAP) and kinetin (KIN) were evaluated and 1-naphthaleneacetic Acid (NAA) and indole-3-butyric acid (IBA) as auxin.

Table 1. Induction medium (M0 to M6) tested for callogenesis of cardoon, with different combinations of cytokinin (BAP and KIN) and auxin (NAA and IBA).

Treatments (mg L^{-1})	M0	M1	M2	M3	M4	M5	M6
Cytokinin	0.5 BAP	2.0 BAP	1.0 BAP	1.0 BAP	2.0 KIN	1.0 KIN	1.0 KIN
Auxin	5.0 NAA	1.0 IBA	1.0 IBA	2.0 IBA	1.0 IBA	1.0 IBA	2.0 IBA

All media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar. The cultures were performed in the same medium every 3 weeks. Cultures were kept in a growth chamber at 25±1°C and 16 h light/8 h dark photoperiod.

Each condition was conducted with four replicates (five explants per replicate), for three genotypes (G7, G8 and G9).

The callus formation (%) was measured at the end of 1st sub-culture. The growth index (GI) of the callus from different treatments was calculated using the equation, GI = [X – X0]/ X0, where X is the volume (mm^3) of the callus at the end of the growth period, and X0 is the volume (mm^3) of the initial explant. The GI was calculated following 2 sub-cultures.

Micropropagation by shoot tips

The offshoots collected in November were surface sterilized, according to Ozsan and Onus (2020b) (DPc). A second protocol (DPd) was tested in January and May assays as follows the protocol DPb described above with minor modification, where the immersion in 70% commercial bleach solution was increased for 20 min. Additionally, an overnight period with running water (W), was tested as W/NoW treatment before the disinfection stage to the offshoots collected in May, and compared with the condition without this treatment (NoW). The shoot tip size was also reduced from 0.1 to 2.5 cm in the first assay (November harvesting) to 0.1-0.3 cm in the second and 0.1-0.8 cm in the third assay (January and May harvesting).

After disinfection, the shoot tips (0.5 cm) from both procedures were aseptically isolated from the offshoots and used as explants. During isolation, offshoots were soaked in an aqueous solution of PVP-40 (0.2 g L^{-1}) to prevent plant material from oxidation.

Sterilized explants were transferred into disposable 10 mL vials (one explant per vial) containing induction medium ¾ MS (Murashige and Skoog, 1962), supplemented with BAP 0.05 mg L^{-1} and IBA 0.005 mg L^{-1}, 3% (w/v) sucrose and 0.7% (w/v) agar. During the multiplication phase, shoots were subcultured every three weeks.

For rooting assays the shots were transferred into rooting medium ¾ MS (RM), supplemented with IBA in different concentrations (10, 15 and 20 mg L^{-1}, denominated in this study as RM10, RM15 and RM20). All media were also supplemented with activated charcoal 1 g L^{-1}, 3% (w/v) sucrose and 0.7% (w/v) agar. Each rooting treatment was conducted with 7-11 replicates (five explants per replicate) according to the condition.

All cultures in the various phases were incubated in a growth chamber in the same conditions as described before.

At the end of the three-week induction phase, the contamination rate and shoot lengths were recorded. The growth index (GI) of the shoots was calculated using the equation, GI = [X – X0]/ X0, where X is the shoot length (mm) at the end of the subculture, and X0 is the shoot length (mm) of the initial explant. The GI was calculated following 2 subcultures.

The rooting rate (%) was also registered after one/two months.

After the rooting phase, plantlets were removed from the culture medium, rinsed under tap water and then transferred to pots filled with peat and grown in a climate chamber under high relative humidity (90%), which was gradually reduced reaching ~35% after 4 weeks. Cultures were kept in a growth chamber at 25±1°C and 16 h light/8 h dark photoperiod.

Statistical analysis

Statistical analysis was performed using SPSS 26.0.0 software. The Kruskal-Wallis test was used to analyze the callus formation and the growth index during callogenesis and to analyze the vigour and root length after two months in micropropagation by offshoots, at a significant level of α=0.05. The means were evaluated by pairwise comparisons test.

Student's t-test was used to analyze the growth index between January versus May, and W/NoW treatment during micropropagation by offshoots, at a significant level of α=0.05.

RESULTS AND DISCUSSION

Plant in vitro culture has been described as having many advantages in the vegetative propagation of disease-free plants (El-Motaleb et al., 2023). Thus, different in vitro techniques have been used for large-scale propagation, and applied in the breeding and conservation of several species (Coelho et al., 2020). These methodologies can also be used to maximize secondary metabolite production, helping the comprehension of its biosynthetic pathway (Aghdasi et al., 2018; Farhan et al., 2018). To our knowledge, this was the first study reporting micropropagation of wild cardoons using two different approaches, callogenesis and micropropagation by shoot tips. Different parameters were analyzed such as harvesting period, disinfection protocols, and culture media.

Callogenesis induction

In the initiation phase of callogenesis, the contamination rate was only 14 and 11% for November and January collection period. However, in May the contamination rate strongly increased to 56%, when the adult leaves were used for callus induction. The type of leaves (young versus adult) seems to be more determinant in initiation than the protocol of material disinfection (DP and DPb) tested (Figure 1).

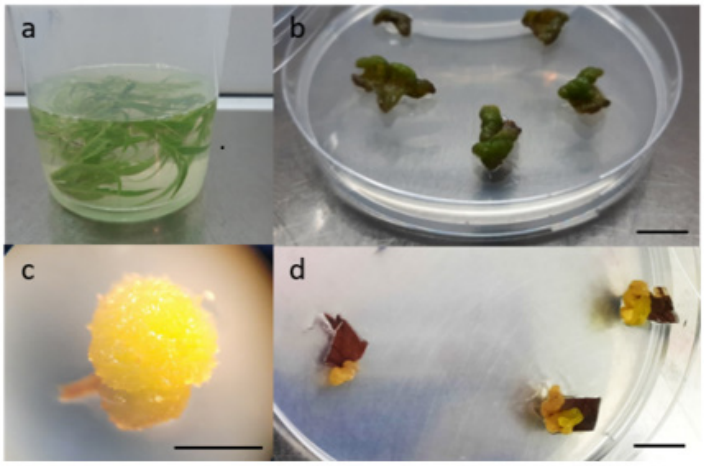

Figure 1. Callus formation from young leaves of cardoon. Leaves during sterilization protocol (a); explant leaves after 4 weeks in culture medium (b); initiation of callus formation (c); callus after 4 weeks (d). Bars: 1 cm.

In all of the seven tested media, it was possible to observe callus formation (Table 2). The percentage of callus formation ranged from 11.7 in M6 medium (KIN:IBA, 1:2 mg L^{-1}) to 26.7 in M0 (BAP:NAA, 1:10 mg L^{-1}) and M3 (BAP:NAA, 1:2 mg L^{-1}) media (Figure 2). But, no significant differences in callus formation were observed between media, at $p \leq 0.05$.

The growth index ranged from 0.47 (M1) to 0.22 (M2) in media supplemented with BAP (Table 2). The cytokinin, kinetin type, was not beneficial for the multiplication of callus, as demonstrated by the null growth index in M4, M5, and M6. Moreover, only in two out of the three tested genotypes callus formation was obtained (Table 3), highlighting the genotype-dependent callogenesis process in cardoon.

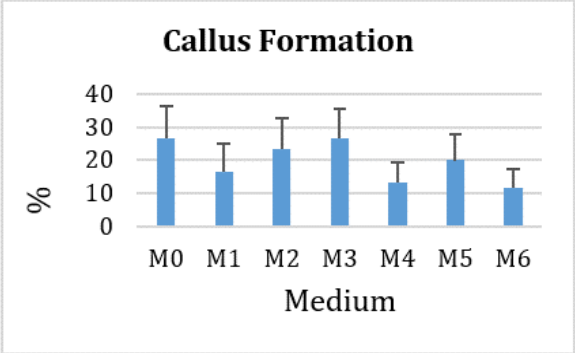

Figure 2. Callus formation (%) obtained in seven different media (M0 to M6). The values are expressed as means ± SE (*n*=12). No significant differences, at p≤0.05.

Table 2. Growth indices assessed across seven distinct media (M0 to M6), with N denoting the number of calluses obtained under each condition.

	M0	M1	M2	M3	M4	M5	M6
Growth Index	0.35 a	0.47 a	0.22 a	0.37 a	0 ab	0 b	0 b
N	20	11	23	19	9	14	14

Significant differences are indicated by different letters, at p≤0.05.

Table 3. Callus formation (mean ± SE) as affected by genotype (G7, G8 and G9); N=28.

Genotype	G7	G8	G9
Callus Formation (%)	32.86±5.46 a	0.00±0.00 b	26.43±5.35 a

Significant differences are indicated by different letters, at p≤0.05.

Cytokinins and auxins promote cell division and their addition is required for callus growth (Abbas et al., 2021). In several species, the callus induction has been dependent on the type and concentration of the cytokinin and auxin combination.

In globe artichoke equal amounts or 10:1 concentrations of auxin: cytokinin, were important for stimulating callogenesis, using BAP and NAA as plant growth regulators (PGR). However, callogenesis has been also reported as a genotype-dependent process (Ozsan and Onus, 2020a). In our study, the type of cytokinin selected was more important than the balance of auxin:cytokinin presented in the culture medium. According to Joshaghani et al. (2014), the callus formation in artichoke was also dependent on PGR presence and explant type, the leaf explant being the best option for callus production. In our study, the kinetin was not beneficial for the multiplication of cardoon callus. On the other hand, Bekheet et al. (2014) describe the highest frequency of callus induction from leaf explants in artichoke, using a culture medium supplemented with KIN instead of BAP.

Micropropagation by shoot tips

The initiation phase of in vitro culture from adult plants collected from the open field is usually a critical step, mainly for species having an underground stem (Bello et al., 2018). In this study, the contamination rate for the initiation step was higher in November (72%) when the DPc disinfection protocol was used. This rate strongly decreased to 19 and 2% in January and May with the DPd protocol.

From the three genotypes tested in January harvesting, the growth index varied from 1.01 to 8.69, after 4 weeks in in vitro culture conditions. However, the oxidation level was high, and the shoot multiplication level was insufficient, whereby only one genotype (G7) was used for further multiplication, rooting and acclimatization assays. Considering the harvesting collection, the growth index of G7 genotype was significantly higher in January (3.53) than in

May (0.29), after 4 weeks in culture. Although GI was higher in W treatment, no statistical difference was observed between treatments (p≤0.05; Table 4).

Table 4. Effect of harvesting period (January and May; *n*=31 to 21), and of the W/NoW treatment (NoW and W; *n*=10 to 11) on the growth index of the G7 genotype, following a 4-week culture period. Significant differences are indicated by different letters, at p≤0.05.

Harvesting period	**January**	**May**
Growth Index	3.53 a	0.28 b
Treatment	**NoW**	**W**
Growth index	0.10 a	0.46 a

Considering the rooting stage, it was possible to obtain plantlet rooting in the three tested conditions. This rate ranged from 2.5 to 10.7% (Figure 2), with RM supplemented with IBA 15 mg L^{-1} having the highest rate, while the lowest rate was obtained in the RM10 medium. No callus formation was also detected in all tested media. The rooted plantlets were acclimatised with success (Figures 3 and 4). In previous studies of in vitro rooting of artichoke plantlets, the rate was also low between 0 to 16% depending on the cultivar, indicating the need for specific optimization for each genotype (Bedini et al., 2012).

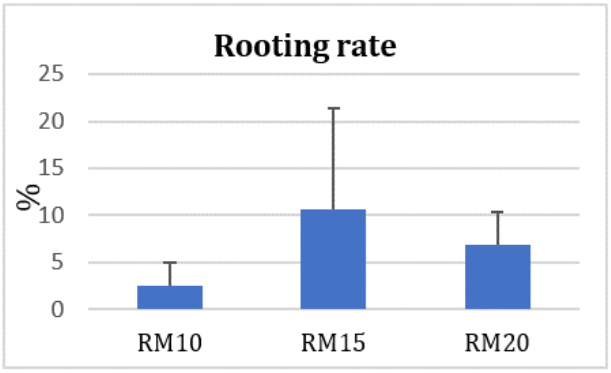

Figure 3. Rooting rate (%) observed in RM10, RM 15 and RM20 of wild cardoon. The values are expressed as means ± SE (*n*=7 to 11). No statistical difference at p≤0.05 was found.

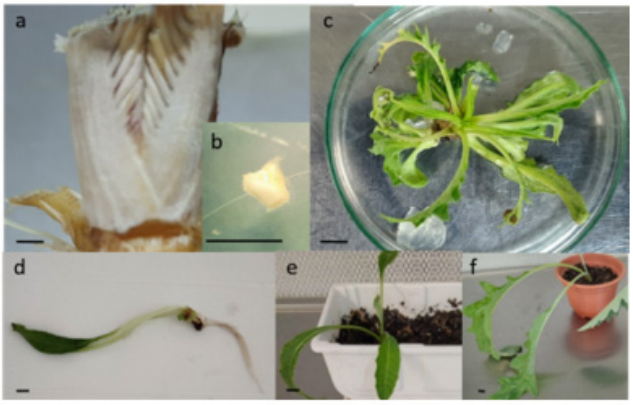

Figure 4. Micropropagation of an adult cardoon plant. Surface sterilized offshoot (a); shoot tip explant (b); shoots in multiplication phase (c); rooted in vitro plant (d); ex-vitro plant with 1 (e) and 2 months (f). Bars: 1 cm.

The major problems detected in this micropropagation system were the level of contamination in the induction stage, phenols exudation conducting to the browning of tissues and the low rooting rate.

CONCLUSIONS

To conclude, a new protocol for callogenesis induction of cardoon genotypes was developed for further indirect organogenesis and secondary metabolites production in aseptic conditions assays. The type of cytokinin (BAP) influenced callus induction. The January period presented the best option for callus formation and shoot induction, while the surface sterilization procedure was very important to initiate the micropropagation by shoot tips. The genotype showed a strong effect in the callus formation and shoot induction, in callogenesis and micropropagation by shoot tips respectively. New protocol optimization will be needed to reduce the oxidation level in shoot explants and to increase the rooting rates during the micropropagation by shoot tips.

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