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IN-VITRO REGENERATION OF *CALENDULA MARITIMA* GUSS. (ASTERACEAE), A THREATENED PLANT ENDEMIC TO WESTERN SICILY

CARRA ANGELA[1], BAMBINA MARCELLA[2], PASTA SALVATORE[1], GARFÌ GIUSEPPE[1], BADALAMENTI ORNELLA[1], CATALANO CATERINA[1], CARIMI FRANCESCO[1]* AND SAJEVA MAURIZIO[2]

[1]*Institute of Biosciences and BioResources (IBBR), National Research Council (CNR), Research Division of Palermo, Corso Calatafimi 414, 90129 Palermo, Italy*
[2]*Department STEBICEF, University of Palermo, via Archirafi 18, 90123 Palermo, Italy*
**Corresponding author's e-mail: francesco.carimi@ibbr.cnr.it; Phone: +39-091-6574578; Fax: +39*-091-423424

Abstract

Calendula maritima is a critically endangered endemic plant of Western Sicily. Besides habitat destruction, the hybridization with the contiguous congener species *C. fulgida* is a major threat to its conservation. For this reason, seed-based propagation and seed storage are not appropriate for conservation purposes. In the present paper we describe a rapid and prolific *In vitro* plant regeneration method by direct organogenesis from leaves of *C. maritima*. Leaf explants were cultured on solid Murashige and Skoog (MS) medium in the presence of several plant growth regulator combinations. The best shoot multiplication rate (2.5 shoots/explant) was obtained on the medium containing 4.4 µM 6-benzylaminopurine in combination with 10 µM ß-naphthoxyacetic acid. Regenerated shoots were successfully rooted on solid MS medium supplemented with several auxins and the best result was obtained with 1.0 µM indole-3-acetic acid (35% of plantlets rooted). Plantlets were thereafter established in the greenhouse (survival frequency 75%) and no phenotypic variations were observed between regenerants and the mother plants.

Key words: Endangered species, *Ex situ* conservation, Organogenesis, Plant regeneration.

Abbreviations: BAP, 6-benzylaminopurine; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; NOA, ß-naphthoxyacetic acid; PGR, plant growth regulators; TDZ, thidiazuron

Introduction

Calendula maritima Guss. (Asteraceae) is a narrow endemic to Western Sicily. It belongs to the *Calendula incana-suffruticosa* species complex (*sensu* Nora *et al.,* 2013), whose distribution range includes Macaronesia (Madeira and Canary islands), Southern Spain, Southern Italy and North West Africa (Morocco, Algeria and Tunisia). Depending on the intensity of summer drought stress, *C. maritima* may behave as an annual or perennial short-lived herb. Because of its adaptive characters it contributes to the biodiversity of nitrophilous and ruderal communities where a large amount of organic nutrients (mainly beached seagrasses and macro-algae and seabird droppings) are present. This plant takes part to different stress-tolerant plant communities linked to rather undisturbed coastal areas, such as rocky or sandy shores, but it is also able to stand quite intense human disturbance thriving also within species-poor hypernitrophilous pioneer assemblages linked to suburban areas. It plays a key-role in the equilibrium of several coastal ecosystems and also in their landscaping. Some populations live within *Crithmo-Limonietea* communities which may be referred to the target habitat type 1240 (Vegetated sea cliffs of the Mediterranean coasts with endemics *Limonium* spp.) of the EU Habitats Directive. Once widespread along the shores of NW Sicily and several islands and islets of Egadi Archipelago, nowadays its distribution is very discontinuous mostly due to habitat fragmentation (Grammatico & Fici, 2008), and several populations have disappeared due to urbanization and coastal degradation. Furthermore, a more worrying threat has been put in evidence in recent times. In the last decade, a study by Plume *et al.* (2013) underlined that hybridization with *Calendula fulgida* Raf., which grows in contiguous areas, could severely affect the fitness of this endangered species.

According to IUCN criteria, this species is considered critically endangered (Troìa & Pasta, 2005). *In situ* conservation is of primary importance but this is not always sufficient to guarantee the survival of a species. Therefore *ex-situ* methods need to be used to ensure protection. In this regard, target 3 of the Global Strategy for Plant Conservation 2008-2014 (Planta Europa, 2008) suggests that "Development of models with protocols for plant conservation and sustainable use based on research and practical experience" are desirable, while among the targets of the Global Strategy 2011-2020 (http://www.cbd.int/gspc/targets.shtml) target 8 indicates as a major challenge the conservation of "at least 75 per cent of threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20 per cent available for recovery and restoration programs".

In the case of *Calendula* seed-based propagation and seed storage are not effective because of the hybridization problems, whereas cuttings are not responsive. Biotechnologies are nowadays considered a useful tool for *ex-situ* plant conservation activities (Bapat *et al*., 2008; Malda *et al*., 1999; Paunescu, 2009) and *In vitro* propagation plays an important role especially in endangered plants conservation (Murashige, 1974; Wochock, 1981; Fay, 1992; Altman & Loberant, 1997; Sarasan *et al*., 2006; Rai, 2010). In line with the GSPC objectives, in this paper we report for the first time a rapid and prolific *In vitro* regeneration method by direct organogenesis from leaves of *C. maritima* as an important step toward its effective conservation.

Material and Methods

Plant material and culture condition: Mature plants of *C. maritima* were collected from three different populations in Sicily, potted in 3.5 lt squared pots filled with soil from the collection sites and cultivated at the collection of IBBR-CNR, UOS of Palermo. Only flowering plants for which it was possible to exclude visible hybridization were collected. Starting material was collected at Trapani seaside, Maraone Islet and Colombaia Islet. Young apical shoots were harvested in springtime. Subsequently, leaves were removed from shoots and the explants were washed in running tap water and cut into nodal segments approximately 1.5 cm in length. Nodal explants were surface sterilized and incubated in Murashige and Skoog (MS) medium (1962) as described by Carra *et al.* (2012). After 4 weeks, axillary shoots (2–3 cm in length) were excised from the stem tissue and subcultured into MS medium for multiplication. Plant material was transferred in Magenta® vessels filled with 40 ml of fresh medium every 20 days.

Adventitious bud regeneration: Leaflet explants were collected from 20-day-old cultures that had been subcultured for at least two *In vitro* propagation cycles. Only the fully expanded leaves without petiole were taken from donor shoots. Leaves were divided into two segments (10-15 mm) consisting in the apical and basal part and placed with the abaxial side in contact with the medium. Cultures were maintained on MS medium added with several plant growth regulators (PGR) as reported in Table 1. PGR were filter sterilized through a 0.22 µm nylon filter and added to the medium after autoclaving. Five leaf explants were placed in each Petri dish and five replicates were done. Cultures were maintained under the same conditions previously described. The effect of each treatment on bud regeneration and growth was determined 60 days after culture initiation by recording the following parameters: the percentages of responsive explants, the percentage of explants with new buds and the mean number of shoots per explants producing shoots.

Rooting and acclimatization: Green shoots (10-20 mm long) were collected at the end of 4th week of *In vitro* culture and incubated in the presence of auxins. Root regeneration was induced on MS medium supplemented with the auxins IAA or IBA each at 0.1 or 1.0 µM. Cultures were incubated under the same culture conditions described above for 4-6 weeks till roots emerged. The effect of PGR was evaluated by recording the percentage of rooted shoots, the number of roots per explant and the average length of the roots. Shoots that formed roots were collected 40 days after rooting treatments and washed with tap water in order to remove the medium before being transplanted individually to Jiffy 7 pots as described in Carra *et al.* (2012).

Data analysis: Ten replicates (Petri dishes) and five explants per replicate were used per treatment (50 explants) and each treatment was repeated twice. The effect of PGR on shoot regeneration and rooting was assessed after 30 and 60 days of culture, respectively. A completely randomized factorial design was utilized. Percent data were arcsine-square-root-transformed for data analysis. The effect of PGR was tested by Analysis of Variance ($p \leq 0.05$) and mean differences were statistically assessed at a 5% level by Tukey's test (SigmaStat 3.5).

Results

Plant production: In order to produce enough plant material for the subsequent *In vitro* experiments, nodal explants (10-20 mm in length) were collected from *In vivo* growing mature plants and introduced *In vitro* on PGR-free MS medium, until enough stock material was regenerated. The percentage of fungal and bacterial contamination varied according the population and the successful percentages ranged from 46% (Trapani seaside) to 82% (Maraone Islet). For this reason and due to its distance from the coast which should strongly reduce any inbreeding risk, only plant material from Maraone Islet was used for the subsequent experiments. For shoot production, nodal segments regenerated *In vitro*, approximately 10 mm in length, were collected from *In vitro*-derived shoots and incubated on PGR-free MS medium in order to produce new shoots for collecting leaf explants for the experiments.

Adventitious bud regeneration: New buds appeared 30 days after incubation at the cut surface of explant (Fig. 1A). Bud regeneration occurred when leaf explants were cultured in the presence of 4.4 µM BAP alone or in combination with 10.0 µM NOA with no significant difference between treatments. The percentage of responsive explants producing new buds ranged from 0 (3.4 µM TDZ + 2.5 µM IBA) to 42 (4.4 µM BAP) and the best multiplication rate in terms of number of regenerated shoots per explant (2.5) was achieved when BAP was used in addition to NOA (Table 1). Our data clearly indicate that BAP alone or in combination with the auxin NOA, was most efficient in bud regeneration compared with TDZ which was not effective in producing new buds (Table 1).

Rooting and acclimatization: Only healthy growing shoots were used for rooting experiments (Fig. 1B). Roots emerged from the cut surface of cuttings about 20 days after treatments. Explants produced roots under all the combinations used with results varying according to the treatment (Table 2). It ranged from 6.6% to 35% (0.1 µM IBA and 1.0 µM IAA, respectively). In terms of number of roots per explant, the higher rate of rooting was achieved with l.0 µM IAA. Under these conditions it was possible to obtain 3.67 roots per explant. The best results for root elongation (17.5 mm) were achieved in the presence of 0.1 µM IBA. Roots emerged without callus formation (Fig. 1C) allowing a successful acclimatization. During acclimatization phase in growth chamber, well-developed plants of *Calendula* were gradually exposed to a lower relative humidity and a higher light intensity. About 40 days after exflasking, plantlets reached about 10 cm in height (Fig. 1D). The best plant acclimatization was obtained with shoots rooted with 1.0 µM IAA. The frequency of plantlet survival during acclimatization and transfer to greenhouse conditions was about 75%.

Table 1. Effect of different growth regulator treatments on callus and shoot formation on leaf explants of *Calendula maritime*.

Medium	Explants forming callus (%)	Explants forming shoots (%)	Mean number of shoots / explants
4.4 µM BAP	56 ± 26^{a}	42 ± 4.7^{a}	1.5 ± 0.4^{a}
4.4 µM BAP + 10 µM NOA	60 ± 30^{a}	35 ± 2.8^{a}	2.5 ± 0.6^{a}
3.4 µM TDZ + 2.5 µM IBA	75 ± 25^{a}	0^{b}	//
Hormone Free	56 ± 4^{a}	0^{b}	//

Data were collected after 60 days from the beginning of the experiment. Means $\pm$ SE, in each column values followed by the same letter are not significantly different at $p \leq 0.05$ level (Tukey's test)

Table 2. Effect of different auxin treatments on roots formation in *Calendula maritima.*

Medium	Rooted shoots (%)	Mean root number /shoot	Mean root length (mm)
0.1 µM IAA	16.7 ± 8.0^{a}	2.0 ± 0.4^{b}	8.5 ± 1.3^{b}
1.0 µM IAA	35.0 ± 12.6^{a}	3.7 ± 0.3^{a}	6.0 ± 1.4^{b}
0.1 µM IBA	6.6 ± 3.8^{b}	1.0 ± 0.2^{b}	17.5 ± 2.5^{a}
1.0 µM IBA	20.0 ± 0.0^{a}	1.3 ± 0.3^{b}	5.0 ± 1.2^{b}

Data were collected 60 days after incubation. Means $\pm$ SE values in each column followed by the same letter are not significantly different at $p \leq 0.05$ level (Tukey's test)

Fig. 1. *In vitro* plant regeneration of *Calendula maritima*. **A** - *In vitro* shoot development on the cut surface of leaf explants cultured in the presence of 4.4 µM BAP and 10 µM NOA. Picture was taken 30 days after initiating cultures. Bar = 1 cm. **B -** Regenerated shoot used for the rooting step. Picture was taken 50 days culture initiation. Bar = 1 cm. **C** - *In vitro* rooting of shoot in the presence of 1.0 µM IAA. Picture was taken 30 days after transfer to auxin medium. Bar = 1 cm. **D -** *In vitro* rooted shoot transferred in a Jiffy pot undergoing acclimatization in a growth chamber. Picture was taken 20 days after the transfer to soil. Bar = 2 cm.

Discussion

The protocol described here was developed to regenerate plants of *C. maritima* starting from leaf explants, and it showed that plant regeneration was possible with BAP alone or in combination with NOA while the others combinations did not produce positive results. To our knowledge this is the first description of a practical *In vitro* plant regeneration protocol for *C. maritima*.

As observed for many other plants (Carimi & De Pasquale, 2003; Duhem *et al.*, 1988; Cassells, 1991; Onay, 2000), *In vitro* regeneration of plantlets from explants collected from adult plants may be difficult or even impossible because of the presence of fungal or bacterial contaminants which limit the introduction of plant material *In vitro*. To avoid *In vitro* contaminations the use of young and actively growing tissues collected in spring was preferred. Similar results were achieved by Leal *et al.* (2009) who obtained 80% of success with *Calendula officinalis* L., starting from nodal segments excised from plants growing under greenhouse conditions. Other authors used several types of explants to

induce *Calendula* regeneration starting from *In vitro* cultured seeds in order to reduce time of axenic culture establishment (Çöçü *et al.*, 2004; Victório *et al.*, 2012; Nikam & Khan, 2014).

Response of cultured explants is strictly dependent on culture condition. PGR influence the ability of the explants to respond to *In vitro* culture conditions. Moreover PGR presence in the culture medium may control frequency of phenotypical and physiological alterations in plants (Ziv, 1991). In our experimental system the best results (in terms of percentage of explants with shoots), were achieved when 4.4 µM BAP, alone or in combination with 10.0 µM NOA, was used. TDZ was not effective in inducing shoot production. These results are in contrast with those reported for *C. officinalis* by Çöçü *et al.* (2004), who obtained the best shoot organogenesis with TDZ in combination with IBA; on the contrary they are consistent with those reported by Victório *et al.* (2012), who stated that TDZ was less effective compared to BAP. Also Bertoni *et al*. (2006) described for *C. officinalis* a micropropagation protocol comparing the performance of BAP, kinetin and zeatin being BAP the most effective in production of new shoots. TDZ is reported to promote shoot proliferation in many species, both woody and herbaceous (Huetteman & Preece, 1993; Schulze, 2007), nevertheless it may inhibit shoot elongation as reported for other species (Arikat *et al.*, 2004; Misic *et al.*, 2006; Carra *et al.*, 2012). In some species, TDZ was ineffective in promoting shoot proliferation e.g. *Cercis canadensis* L. (Yusnita *et al.*, 1990), *Gymnocladus dioicus* (L.) K. Koch (Smith & Obeidy, 1991) and *Vitis rotundifolia* Michx. (Gray & Benton, 1991), and also had negative effects mostly in prolonged exposures (Kim *et al.*, 1997). Large-scale vegetative propagation of herbaceous species depends on their rooting activity, which is affected by many factors (De Klerk, 2002). Rooting was attempted with IBA and IAA, both supplemented at 0.1 and 1.0 µM. Rooting was achieved under all culture conditions, but with significant differences. The highest percentage of rooted plants and mean root number per rooted plant were recorded with IAA at 1.0 µM. Moreover when IBA was used, callus formation was observed at the cut surface which is undesired because roots could have been induced from callus cells, producing an inappropriate root system for *ex vitro* transfer, as reported for other species (Ricci & Bertoletti, 2008).

In conclusion, here we report for the first time a reliable procedure to propagate *C. maritima* starting from leaf explants. Our experimental procedure allows the production of a high number of individuals independently from the natural vegetative cycle in the wild, and can be successfully used to increase the production of new specimens for *ex situ* conservation purposes. Further investigations to confirm genetic stability are planned to evaluate the possibility to use regenerated plants for reintroduction and reinforcement in natural habitats.

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References

Altman, A. and B. Loberant. 1998. Micropropagation: clonal plant propagation *In vitro*. In: *Agricultural biotechnology*, (Ed.): A. Altman, Marker Dekker, New York, pp. 19-42.

Arikat, N.A., F.M. Jawad, N.S. Karam and R.A. Shibli. 2004. Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa*). *Sci. Hortic.,* 100: 193-202.

Bapat, V.A., S.R. Yadav and G.B. Dixit. 2008. Rescue of endangered plants through biotechnological applications. *Natl. Acad. Sci. Lett.,* 31: 201-210.

Bertoni, B.W., C.F. Damião Filho, J.R. Moro, S.C. França and A.M.S. Pereira. 2006. Micropropagação de *Calendula officinalis* L. *Revista Brasileira de Plantas Medicinais* 8: 48-54.

Carimi, F. and F. De Pasquale. 2003. Micropropagation of citrus. In: *Micropropagation of woody trees and fruits*. Springer Netherlands, pp. 589-619.

Carra, A., M. Sajeva, L. Abbate, M. Siragusa, F. Sottile and F. Carimi. 2012. *In vitro* plant regeneration of caper (*Capparis spinosa* L.) from floral explants and genetic stability of regenerants. *Plant Cell Tiss. Org. Cult.,* 2: 373-381.

Cassells, A.C. 1991. Problems in tissue culture: culture contamination. In: *Micropropagation* Springer Netherlands pp. 31-44.

Çöçü, S., S. Uranbey, A. İpek, K.M. Khawar, E.O. Sarihan, M.D. Kay, I. Parmaksiz and S. Özcan. 2004. Adventitious shoot regeneration and micropropagation in *Calendula officinalis* L. *Biol. Plant.,* 48: 449-451.

De Klerk, G.J. 2002. Rooting of microcuttings: theory and practice. *In Vitro Cell Dev. Biol. Plant.,* 38: 415-422.

Duhem, K., N. Le Mercier and P. Boxus. 1989. Donnés nouvelles sur l’induction et le développement d’embryos somatiques chez *Theobroma cacao* L. *Café Cacao Thé* 33: 9-14.

Fay, M.F. 1992. Conservation of rare and endangered plants using *In vitro* methods. *In Vitro Cell Dev. Biol. Plant.,* 28: 1-4.

Grammatico, F. and S. Fici. 2008. Distribuzione, fenologia e *status* di conservazione di *Calendula suffruticosa* Vahl subsp. *maritima* (Guss.) Meikle (Asteraceae). *Naturalista Sicil.,* 32: 305-318.

Gray, D.J. and C.M. Benton. 1991. *In vitro* micropropagation and plant establishment of muscadine grape cultivars (*Vitis rotundifolia*). *Plant Cell Tiss. Org. Cult.,* 27: 7-14.

Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss Org. Cult.,* 33: 105-119.

Kim, M.K., Sommer H.E., Bongarten B.C. and S.A. Merkle. 1997. High frequency induction of adventitious shoots from hypocotyl segments of *Liquidambar styraciflua* L. by thidiazuron. *Plant Cell Rep.,* 16: 536-540.

Leal, F., A. Rodrigues, D. Fernandes, F.M. Nunes, J. Cipriano, J. Ramos, S. Teixeira, S. Vieira, L. Carvalho and O. Pinto-Carnide. 2009. *In vitro* multiplication of *Calendula arvensis* for secondary metabolites extraction. *Acta Hort.,* (ISHS) 812: 251-256.

Malda, G., R. Backhaus and C. Martin. 1999. Alterations in growth and crassulacean acid metabolism (CAM) activity of *In vitro* cultured cactus. *Plant Cell Tiss Org. Cult.,* 58: 1-9.

Mišić, D., D. *Grubišić* and R. Konjević. 2006. Micropropagation of *Salvia brachyodon* trough nodal explants. *Biol. Plant*., 50: 473-476.

Murashige, T. 1974. Plant propagation through tissue culture. *Ann. Rev. Plant Physiol.,* 25: 135-166.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.,* 15: 473-497.

Nikam, S.L. and S.J. Khan. 2014. Efficacy of growth hormone for callus induction in *Calendula officinalis* L. *J. Cell Tiss. Res.,* 14: 4153-4157.

Nora, S., S. Castro, J. Loureiro, A.C. Gonçalves, H. Oliveira, M. Castro, C. Santos and P. Silveira. 2013. Flow cytometric and karyological analyses of *Calendula* species from Iberian Peninsula. *Plant Syst. Evol.,* 299: 853-864.

Onay, A. 2000. Micropropagation of pistachio from mature trees. *Plant Cell Tiss. Org. Cult.,* 60: 159-163.

Paunescu, A. 2009. Biotechnology for endangered plant conservation: a critical overview. *Rom Biotech. Lett.,* 14: 4095-4103.

Planta Europa. 2008. *A Sustainable Future for Europe; the European Strategy for Plant Conservation 2008–2014*. Plantlife International (Salisbury, UK) and the Council of Europe (Strasbourg, France).

Plume, O., A. Troìa and F.M. Raimondo. 2013. Hybridization and competition between the endangered sea marigold (*Calendula maritima*, Asteraceae) and a more common congener. *Plant Biosyst.,* 149: 68-77.

Rai, M.K. 2010. Review: biotechnological strategies for conservation of rare and endangered medicinal plants. *Biodiversitas* 11: 157-166.

Ricci, A. and C. Bertoletti. 2008. Urea derivatives on the move: cytokinin-like activity and adventitious rooting enhancement depend on chemical structure. *Plant Biol.,* 11: 262-272.

Sarasan, V., R. Cripps, M.M. Ramsay, C. Atherton, M. McMichen, G. Prendergast and J.K. Rowntree. 2006. Conservation *In vitro* of threatened plants: progress in the past decade. *In Vitro Cell Dev. Biol. Plant.,* 42: 206-214.

Schulze, J. 2007. Improvements in cereal tissue culture by thidiazuron: a review. *Fruit Veg. Cereal Sci. Biotechnol.,* 1: 64-79.

Smith, M.A.L. and A. Obeidy. 1991. Micropropagation of a mature, male Kentucky coffee tree. *Hort. Sci., 2*6: 1426-1426.

Troìa, A. and S. Pasta. 2005. *Calendula maritima.* In: (Eds.): Montmollin B. (de) and Strahm W. *The Top 50 Mediterranean Island Plants, Wild plants at the brink of extinction, and what is needed to save them*. IUCN/SSC Mediterranean Islands Plant Specialist Group, IUCN, Gland, Switzerland and Cambridge, pp. 92-93.

Victório, C.P., C.L.S. Lage and A. Sato. 2012. Tissue culture techniques in the proliferation of shoots and roots of *Calendula officinalis*. *Rev. Cienc. Agron.,* 43: 539-545.

Wochok, Z.S. 1981. The role of tissue culture in preserving threatened and endangered plant species. *Biol. Conserv.,* 20: 83-89.

Yusnita, S., R.L. Geneve and S.T. Kester. 1990. Micropropagation of white flowering eastern redbud (*Cercis canadensis* var. *alba* L.). *J. Environ. Hortic.,* 8: 177-179.

Ziv, M. 1991. Vitrification: morphological and physiological disorders of *In vitro* plants. In: *Micropropagation*. Springer Netherlands, pp 45-69.

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