

# In Vitro Callogenesis and *Agrobacterium*-Mediated Transformation of Globe Artichoke

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**Keywords:** globe artichoke, callus culture, genetic transformation, *Agrobacterium tumefaciens*

## Abstract

Micropropagation techniques have been widely applied in globe artichoke (*C. cardunculus* L. var. *scolymus*), however, efficient protocols for the establishment of in vitro callogenesis and organogenesis, a pre-requisite for *Agrobacterium*-mediated genetic transformation, have not been set up so far. We developed an efficient protocol for callus induction from leaf explants of three globe artichoke genotypes of the varietal type 'Romanesco'; after just one week culture callus formed with an efficiency close to 100%. Leaf explants were transformed with *Agrobacterium tumefaciens* Ag10 01-124 strain, harboring the binary vector pCAMBIA 2301 with the gene marker GUS, under the control of CaMV 35S promoter. After two weeks, about 30% of the calli obtained from infected leaf explants were positive to GUS assay.

## INTRODUCTION

Globe artichoke (*Cynara cardunculus* var. *scolymus* L.) is an herbaceous perennial plant native to the Mediterranean and whose cultivation is widely distributed all over the world, even though it is mainly concentrated in Mediterranean countries. In recent years the increasing demand for functional foods has led to a renewed interest in this crop, which is one of the richest natural sources of bioactive phenolic compounds, like caffeoylquinic acids (CQAs).

Micropropagation techniques have been well established for clonal propagation of globe artichoke (Ancora, 1986; Barba et al., 2004; Cadinu et al., 2004), on the other hand the regeneration of adventitious shoots from somatic tissues has been only occasionally reported (Ordas et al., 1990, 1991; Kchouk et al., 1997; El-Bahr et al., 2001). The difficulty in obtaining the induction of in vitro callogenesis and the following regeneration of a whole plant from the callus, hampers the possibility to generate stable genetically transformed globe artichoke plants. (Ordas et al., 1991; Kchouk et al., 1997).

Here we report on the setting up of an efficient protocol for the induction of in vitro callogenesis from globe artichoke leaf explants as well as on the obtainment of stable genetically modified calli from leaf explants infected with *Agrobacterium tumefaciens*.

## MATERIALS AND METHODS

### In Vitro Culture and Callogenesis

The starting materials were virus-free plantlets, obtained via meristem tip culture (Barba et al., 2004; Cadinu et al., 2004) of three globe artichoke clones (C3-RPO, C3-RR and SAROM) of the varietal type 'Romanesco'. The plantlets were grown on MS (Murashige and Skoog, 1962) medium including vitamins and BAP (2,22 µM), pH 5.7, 23°C. On the whole 108 combinations of light/dark exposures (photoperiod of 16 h light/8 h darkness, 48 h darkness after 16 h light/8 h darkness and full darkness), hormone

concentrations (BAP, IBA and NAA ranged from 0.5 to 3.0 mg/L) without or with additional compounds (glutathion 200 mg/L, ascorbic and citric acid 5 mg/L, activated charcoal 0,2%, polyvinylpyrrolidone 0,25% and silver nitrate 5 µM) were assessed for each genotype. Each combination was made in two replicates with an average of 8-10 leaf explants in each plate.

### ***Agrobacterium*-Mediated Transformation**

The strain AGL0 01-124 of *A. tumefaciens* containing the pCAMBIA 2301 (see <http://www.cambia.org>) binary vector was used for genetic transformation. The bacterium was cultured in 2 ml of LB medium (Bertani, 2004), including 50 mg/L kanamycin and 50 mg/L rifampicin at 28°C. Leaf explants were dipped in *Agrobacterium* inoculation medium (MS 4,4 g/L, sucrose 30 g/L, tryptone 500 mg/L supplemented with 100 mM acetosyringone, pH 5.8) for 15 min. After blotting on sterile filter paper, the infected explants were cultured on the co-cultivation medium (MS 4,4 g/L, sucrose 30 g/L, casein 800 mg/L supplemented with 100 mM acetosyringone, 1 mg/L BAP and 3 mg/L NAA, pH 5.8) for 2 days in darkness at 25°C. Infected explants were transferred onto a selection medium (MS 4,4 g/L, sucrose 30 g/L, casein 800 mg/L supplemented with 75 mg/L kanamycin, 250 mg/L cefatoxime, 50 mg/L vancomycin, 1 mg/L BAP and 3 mg/L NAA, pH 5.8) and cultured in darkness at 25°C. The explants were transferred to a fresh selection medium every 20 days. The kanamycin-resistant calli were assayed by GUS-staining and incubated at 37°C for 12 h.

## **RESULTS AND DISCUSSION**

### **In Vitro Culture and Callogenesis**

Browning is often observed in tissue cultures and may be influenced by factors such explant tissue, type and composition of the medium and culture conditions (JunHui et al., 2000) as well as phenolic compounds and phenol oxidase activity. Globe artichoke leaves are highly rich in phenolics (Wang et al., 2003; Schutz et al., 2004; Frattiani et al., 2007; Lattanzio et al., 2009), which caused enzymatic browning in leaf explants. In order to avoid this phenomenon, leading to tissue necrosis, two strategies were adopted: (i) keeping the explants in full darkness, since exposure of the explants to light condition has been shown to increase the synthesis of polyphenols in the leaves (Moglia et al., 2008), (ii) addition in the media of polyphenol adsorbent compounds (activated charcoal, silver nitrate and polyvinyl pyrrolidone) as well as polyphenol oxidase inhibitors (glutathion, ascorbic acid and citric acid). A total of 108 combinations of light/dark exposure, hormone ratios, provision of polyphenol adsorbent compounds and polyphenol oxidase inhibitors were tested on three globe artichoke genotypes, belonging to the 'Romanesco' varietal groups: C3-RPO, C3-RR, and SAROM. Despite different combination of BAP, IBA and NAA used in the media, when the explants were kept in full darkness the percentages of callus formation reached 100% in the 'C3-RPO' genotype and about 80% in 'C3-RR' and 'SAROM' (Table 1). Callogenesis induction under photoperiod conditions (16 h light/8 h darkness) was lower than the one obtained in full darkness and decreased after 2 weeks of culture (data not shown). The best performance was obtained with the medium containing 1 mg/L BAP and 3 mg/L NAA, in full darkness (Fig. 1). Among the additional compounds tested in the media, supplementation of citric acid and ascorbic acid favoured the prevention of callus necrosis under light condition, even after three weeks of culture (data not shown).

### ***Agrobacterium*-Mediated Transformation**

After two weeks, the calli developed from leaf explants, transformed with *A. tumefaciens* strain AGL0 01-124 containing pCAMBIA 2301 vector, were subjected to histochemical analysis, and about 30% of them were positive to GUS assay. Not-infected calli, cultured on medium with (+Km) and without antibiotics (-Km), were also stained for GUS activity and no blue spots were detectable. The binary vector pCAMBIA 0321

contains a catalase gene which prevents its expression in *Agrobacterium* cells, but ensures its specific expression in plant cells (see <http://www.cambia.org>; Chen et al., 2006), thus the GUS activity detected in calli was symptomatic of the occurrence of stable transformation. However, the detection of blue spots in the calli highlighted that stable transformation took place unevenly in the tissues (Fig. 2).

## CONCLUSIONS

The production of secondary metabolites using plant cells and cell tissue systems has been the subject of extended research. We have developed an efficient protocol for the production of calli of globe artichoke, a species exceptionally rich in secondary metabolites of nutraceutical and pharmaceutical interest, whose synthesis may be induced 'in vitro' by precursor feeding elicitors and/or may be stimulated by nutritional stresses.

Furthermore, the development of a protocol for the obtaining of transgenic calli open the way for functionally characterizing genes involved in the biosynthetic pathway of secondary metabolites as well as for allowing the over-expression of genes playing a key role in their synthesis.

## ACKNOWLEDGEMENTS

The authors kindly thank Prof. M. Barba and Dr. P. Belligoli from CRA Istituto Sperimentale per la Patologia Vegetale, Roma and Dr. M. Cadinu from Agris Sardegna, Cagliari for providing plant material.

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## **Tables**

Table 1. Percentages of callus formation, after 2 weeks of culture, in the Romanesco varietal clone C3-RPO, C3-RR and SAROM. Combinations of light/dark (light:16 h light/8 h; dark: full darkness) with different concentration of hormones (BAP, IBA and NAA) were tested for each of the genotype in study.

Light/dark	BAP (mg/L)	IBA (mg/L)	NAA (mg/L)	% calli		
				C3-RPO	C3-RR	SAROM
Light	0,5	1	0,5	83,3	28,6	28,6
Dark	0,5	1	0,5	100	78	80
Light	1	1,5	1	66,7	33,3	50
Dark	1	1,5	1	100	55,5	37,5
Light	2	1	2	33,3	16,7	44,4
Dark	2	1	2	83,3	75	77,8
Light	1	-	3	87,5	60	63,6
Dark	1	-	3	100	100	100

**Figures**

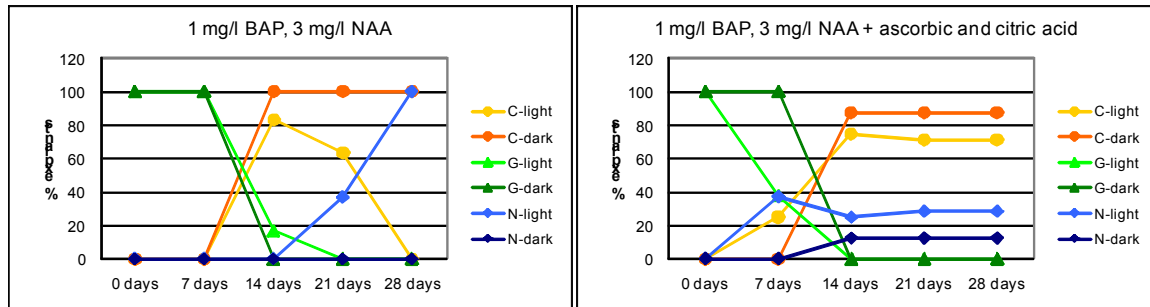


Fig. 1. Graphs show the percentage of globe artichoke ‘Romanesco’ SAROM explants (axis Y) during four weeks (axis X) grown in the media described above each graph. G: explants still green; N: necrotic explants; C: explants forming callus.

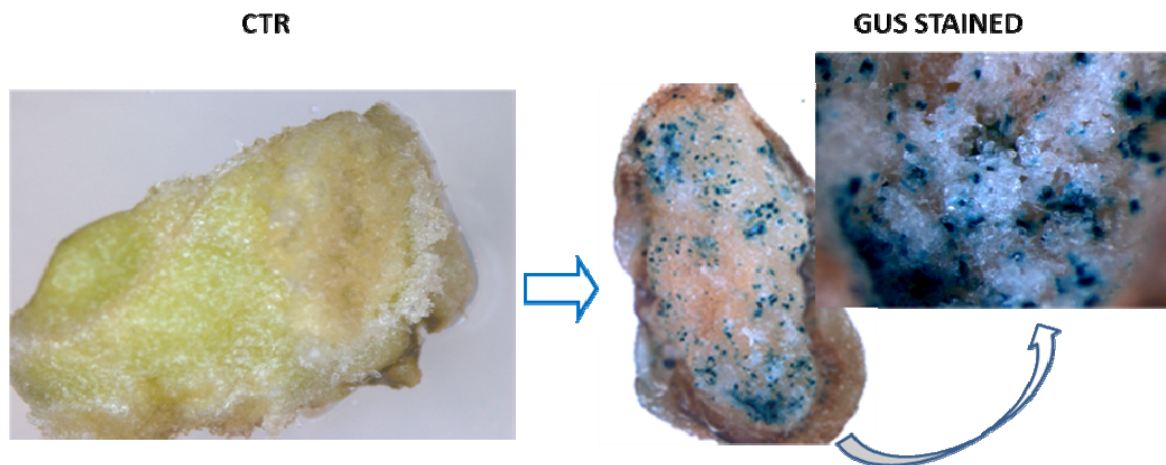


Fig. 2. Transformed calli subjected to histochemical analysis. Blue spots indicate GUS gene expression.

