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A validated slow-growth *in vitro* conservation protocol for globe artichoke germplasm: A cost-effective tool to preserve from wild to elite genotypes

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ABSTRACT

A reliable and reproducible genotype-independent protocol for slow-growth storage of globe artichoke was established for the first time to meet two needs: genetic resources conservation and labour costs reduction in commercial laboratories. Growth reduction was achieved by supplementing osmotic agents to the media. Plant responses to *in vitro* storage, genetic stability and field performance were the parameters used to evaluate the germplasm conservation conditions. Forty-nine treatments were applied, as the result of seven genotypes with seven media. After 12 months of storage, culture survival across genotypes ranged from 65% to 85% and all the media tested supported 100% regrowth. All genotypes regained their full growth potential within two months. Genetic stability between mother plants grown in the field and *in vitro* conserved plantlets at 6 and 12 months of storage was assessed by molecular markers to identify the most suitable storage media.

Protocol suitability was validated by a field test using an approved list of plant descriptors for globe artichoke. Morphological data highlighted that the slight genetic instability detected by molecular markers did not affect significantly plants morphology and their agronomic traits.

The results indicate that the minimal growth medium of choice for globe artichoke conservation is the one where the seven genotypes displayed phenotypes similar to the control, coupled with the lowest percentage of changes (2.43%) at a molecular level. As far as we know there are no published reports on *in vitro* conservation protocolfor globe artichoke applied to different genotypes and validated by an appropriate field test.

1. Introduction

Native to the Mediterranean basin, the globe artichoke (*Cynara cardunculus* var. *scolymus* L.) is widely grown around the world. Italy and Spain are the world's leading producers followed by France (FAO STAT 2012; http://faostat.fao.org).

Italy harbours the richest primary cultivated artichoke gene pool, and houses the most abundant *in situ* diversity (Bianco, 1990). Globe artichoke is the third largest vegetable crop cultivated in Italy, after tomato and potato.

Interest in this species has recently increased along with its new possible uses. Globe artichoke is commercially important for its dietary and pharmaceutical value (Saènz Rodriguez et al., 2002; Coinu et al., 2007; Fantini et al., 2011). It is also exploited for oil production from seeds (Foti et al., 1999), inulin from roots (Raccuia and Melilli, 2010) as well as for energy from biomass (Ierna and Mauromicale, 2010; Ierna et al., 2012). It is thus crucial to gain access to a wide range of plant genetic resources that provide varied traits.

As globe artichoke is a highly heterozygous species, it leads to a segregating progeny and considerable variation in seed types. As a result, its germplasm cannot be stored effectively by conventional means. To preserve the genetic integrity, selected genotypes are maintained vegetatively (Barbieri, 1967; Snyder, 1979) and

Abbreviations: ANOVA, one-way analysis of variance; IBA, indole 3 butyric acid; ISSR, inter-simple sequence repeats; KIN, kinetin; NAA, 1-naphthaleneacetic acid; PIC, polymorphic information content; SSR, simple sequence repeats.

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propagated as genotypes Traditionally, the field collection of globe artichoke is the most widely used storage method, and provides the possibility of continuously characterizing and evaluating the germplasm. Although field collection provides easy access to conserved material, its maintenance is time-consuming, it requires a lot of space and is labour-intensive. In addition, losses of germplasm maintained in the field collection can occur due to environmental and biological hazards (Babes et al., 2004; Bianco, 1990). Nowadays, only limited amounts of artichoke germplasm are conserved in field collections, which are mainly carried out by public institutions.

Strict international rules have established the sanitary and qualitative requirements for safe germplasm exchange [Directives 93/61/CEE and 93/62/CEE (OJ L250, 07/10/1993 pp. 19–30)]. Therefore, the exchange of plant material is becoming increasingly difficult due to the great risk of transferring disease. Alongside field practices, propagation through *in vitro* meristem culture has proved to be a safe and more practical method for the collection, multiplication and conservation of valuable globe artichoke germplasm. This technique also leads to the production of large-scale, phenotypically homogeneous and disease-free plants, in particular for spring cultivars (De Leo and Greco, 1973; Ancora et al., 1981; Pecaut and Dumas de Vaulx, 1983; Rossi and De Pauli, 1992).

In Italy, most commercial cultivars are the result of the *in vitro* isolation of selected individuals during propagation (Ancora, 1986; Ancora and Saccardo, 1987). The extensive use of this propagation method has led to the conservation of genetic resources, as well as the large-scale production and commercialization of superior genotypes by private tissue culture laboratories. More than four million micropropagated artichoke plants were produced in Italy in 2010, to provide propagation material to farmers (Lambardi and Previati, 2012). This has led, over time, to both the substitution of local genotypes for improved uniform varieties and changes in growing techniques. In turn, it causes the possible erosion of globe artichoke genetic resources and the loss of valuable material.

Since 2007, artichoke landraces have been collected, characterized and preserved in order to conserve these gene pools both in field and *in vitro* collections (CYNARES EU project, Pagnotta, 2012). The high rates of multiplication achieved by micropropagation and the frequency of sub-cultures do not match well with the management of large *in vitro* collections as this would result in increased costs and the risk of contamination leading to losses of genetic material (Rey et al., 2013). Micropropagation has thus been considered only for the short-term storage of globe artichoke germplasm.

Various *in vitro* conservation methods can be employed, depending on the use and storage duration required (Engelmann, 1997, 2011). To establish globe artichoke gene-bank facilities, the two most widespread strategies are slow-growth systems and cryopreservation. Cryopreservation was recently carried out for globe artichoke (Tavazza et al., 2013), and can be used for the long-term conservation of germplasm, but not for its distribution.

The establishment of a medium-term conservation protocol would be useful for international germplasm distribution and, as a complementary option, for the safer conservation of the globe artichoke germplasm (Withers and Engelmann, 1998). This method of storing the germplasm could also be beneficial in assisting the micropropagation industry to minimise the costs of commercial under-production. In fact, in a commercial tissue culture laboratories there is no year-round demand for young plants but there are peaks in delivery and labour.

Our result are particularly innovative since in terms of the current state of knowledge, only two papers have reported the slow-growth conservation of globe artichoke (Bekheet, 2007; Benelli et al., 2010), while to the best of our knowledge no data on the field performance of globe artichoke plants after retrieval from storage conditions are available.

The main purpose of *ex situ* gene-banks is to maintain the integrity and functionality of stored samples. The maintenance of the true-to-type nature of *in vitro* plants is an important requisite to uphold certain agronomic and horticultural traits when using elite genotype, not just for conservation, but also for commercial purposes. Genetic stability in tissue culture has long been a concern in the application of *in vitro* techniques for the conservation of crop germplasm. This is why there is great interest in techniques that can determine whether the material retrieved from *in vitro* conservation is genetically identical to the material accessed.

Molecular markers are being increasingly used to monitor genetic variations and germplasm identification (Mondini et al., 2009). For conservation purposes they have been efficiently used to detect genetic stability in potato by AFLP (Aversano et al., 2011), in *Hydrangea macrophylla* by ISSR (Inter-Simple Sequence Repeats) (Liu et al., 2011), and in guava and globe artichoke by both SSR (Simple Sequence Repeats) and ISSR markers (Rai et al., 2012; Rey et al., 2013). The number of molecular markers available in globe artichoke has increased considerably and we successfully set a series of feasible markers to be used to characterize globe artichoke in order to accurately discriminate between genotypes (Crinò et al.,2008; Boury et al., 2011; Ciancolini et al., 2012; Rey et al., 2013).

The aim of this study was to devise a simple and reliable method for the medium-term storage of globe artichoke cultures by slowing down growth by osmotic stress without altering the germplasm's genetic stability. The best storage parameters for slow growing artichoke germplasm were defined in terms of their genetic and field performance once retrieved from storage.

2. Material and methods

2.1. Plant material

Seven Italian spring Romanesco globe artichoke genotypes were considered: S2, S3, S8, S11, S17, S18, S23. These were selected from a set of 19 agronomically interesting genotypes isolated from traditionally cultivated landraces (Crinò et al., 2008) and subsequently characterized (Ciancolini et al., 2012).

Shoot explants, 2–3 cm in length, were collected from 6-year-old *in vitro* plants from a germplasm collection kept through monthly subculture on a Gik basal medium (Tavazza et al., 2004), supplemented with 2 mg l^{-1} Kinetin (KIN), 0.1 mg l^{-1} indole-3-butyric acid (IBA), 3% sucrose and 7 g l^{-1} Plant agar, pH 5.8 and maintained at 18 ± 2 °C under a 16-h light photoperiod provided by cool-white fluorescent lamps (37.5 μE m^{-2} s^{-1}). The same environmental conditions were applied in slow-growth experiments. For each genotype, plantlets were further multiplied by axillary branching at 4–5 week intervals to establish stock cultures.

2.2. In vitro storage conditions

To slow down the growth of *in vitro* plants, thus extending the time between subcultures, the carbohydrate level in the Gik medium was increased by replacing 3% sucrose with different combinations and concentrations of sucrose plus mannitol or sorbitol. Altogether two concentrations of sucrose (2 and 3% w/v) with either mannitol (4% w/v) or sorbitol (2 and 4% w/v) were tested. Forty-nine treatments were applied, as the resulted combination of seven genotypes with seven media. Explants (about 1 cm long) from micropropagated plants were: (a) maintained through monthly sub-culture on Gik medium as a control, hereafter referred to as medium T0; (b) stored on Gik medium (T1) or on modified Gik medium containing 30 g l^{-1} sucrose and 40 g l^{-1} mannitol (T2); 30 g l^{-1} sucrose and 40 g l^{-1} sorbitol (T3); 30 g l^{-1} sucrose and 20 g l^{-1} sorbitol (T4); 20 g l^{-1} sucrose and 40 g l^{-1} sorbitol (T5); or

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