Scientia Horticulturae 130 (2011) 681–690

Contents lists available at SciVerse ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Increased regeneration capacity in spinach lines obtained by *in vitro* self-fertilisation

J. Milojević[a], Lj. Tubić[a], S. Zdravković-Korać[a,*], I. Dragićević[b], D. Ćalić-Dragosavac[a], B. Vinterhalter[a]

[a] University of Belgrade, Institute for Biological Research "S. Stanković", Despot Stefan Blvd. 142, 11 060 Belgrade, Serbia
[b] University of Belgrade, Faculty of Biology, Studentski trg 16, 11 060 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 13 March 2011
Received in revised form 1 July 2011
Accepted 17 August 2011

Keywords:
Amaranthaceae
Genotype
In vitro self-fertilisation
Somatic embryogenesis
Spinacia oleracea
Tissue culture

ABSTRACT

Somatic embryos (SEs) were induced from apical sections of the lateral roots of spinach seedlings (1 cm), which were cultivated on solid Murashige and Skoog (MS) medium with 20 μM α-naphthaleneacetic acid and 5 μM gibberellic acid. Apical shoots of the same lines were isolated and cultivated on plant growth regulator-free medium. The regeneration capacities of seedlings randomly chosen from a population were quite low and variable, and only 4 out of 30 lines responded at the frequency of 85–100%, with 6.96–9.96 SEs per explant and up to 347 SEs per seedling over a 12-week period. These SEs were isolated and maintained on medium with 5 μM kinetin. Plants derived from seedlings' apical shoot and SEs self-fertilised *in vitro* and set seeds, and these seedlings (S_1) were used to induce regeneration. Similarly, S_2–S_4 seedlings were obtained and the regeneration capacities of 23 S_1, 23 S_2, 17 S_3 and 5 S_4-seedlings were compared to parental lines. Of these, four S_3 and S_4 lines with extremely high regeneration capacities were selected. These lines exhibited 78–139 fold higher embryo-forming capacities than the mother plant, and produced 38.9–68.3 SEs per explant and 1339–2181 SEs per seedling during the same time period. In addition, the process of somatic embryogenesis began 2–4 weeks earlier in these lines, and root explants taken from SE-derived plants of these lines retained high and stable regeneration capacities, and therefore may be ideal material for genetic transformation.

1. Introduction

Since the first report in 1973 (Nešković and Radojević, 1973), when only one regenerating callus was obtained, spinach was believed to be recalcitrant to *in vitro* regeneration. In the decades that followed, a number of papers dealing with the *in vitro* regeneration of spinach appeared, and it became increasingly clear that genotype is one of the most important factors affecting the regeneration capacity of spinach (Al-Khayri et al., 1991; Komai et al., 1996; Knoll et al., 1997; Zhang and Zeevaart, 1999). In fact, the poor results obtained during early attempts at *in vitro* regeneration may be explained by the low regeneration capacity of the 'Matador' cultivar used. Although later we were able to achieve better results with this cultivar (Zdravković-Korać and Nešković, 1998, 1999), its regeneration capacity remains rather low, and significantly better results were achieved using other spinach cultivars, such as: 'Nippon' (Ishizaki et al., 2001), 'Longstanding' (Knoll et al., 1997, Zhang and Zeevaart, 1999), 'Carpo' (Xiao and Branchard, 1995), 'Jiromaru', 'Nihon' and 'Hoyo' (Komai et al., 1996). However, in addition to the high variability observed in the regeneration capacity of different spinach cultivars, high individual variability within a population of the same spinach cultivar has been reported (Ishizaki et al., 2001), in agreement with similar findings in melons (Molina and Nuez, 1995) and alfalfa (Chen et al., 1987). In fact, significant regeneration capacity differences among both cultivars and inbred lines were found in other species as well, such as: potatoes (Seabrook and Douglass, 2001), soybeans (Kita et al., 2007), sugarcane (Basnayake et al., 2011), sunflowers (Nestares et al., 2002), sugar beets (Zhang et al., 2008), wheat (Ozbay and Özgen, 2010), rye (Popelka and Altpeter, 2003) and maize (Rakshit et al., 2010). Furthermore, Armstrong et al. (1992) was able to increase the regeneration capacity of an elite inbred maize line (from 0.2% to 46%) through backcross breeding, while Kumar et al. (1998) was able to obtain somatic embryos in hybrids between completely unresponsive and highly responsive cotton lines. Rose et al. (1999) obtained highly embryogenic *Medicago truncatula* line 'Jermalong 2HA' from the poorly embryogenic 'Jermalong' cultivar, by a cycle of tissue culture followed by a number of seed generations and continued selection from the high regenerators.

Interestingly, Wan et al. (1988) identified two complementary genes which they proposed to be responsible for *in vitro* regeneration in alfalfa, in agreement with subsequent findings in melons (Molina and Nuez, 1996) and *Cyclamen persicum* (Pueschel et al., 2003). In addition, genetic control of *in vitro* morphogenesis has

* Corresponding author. Tel.: +381 11 2078 366; fax: +381 11 2761 433.
E-mail addresses: koracs@vektor.net, szk@ibiss.bg.ac.rs (S. Zdravković-Korać).

0304-4238/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2011.08.024

been confirmed in a number of species, while 1–8 loci controlling the genetic variation of tissue culture response were revealed by QTL mapping (for review see Bolibok and Rakoczy-Trojanowska, 2006). Furthermore, Mitić et al. (1999) and Pinto et al. (2008) suggested the involvement of maternal effects in the inheritance of regeneration capacity in wheat and *Eucalyptus globulus*, respectively. All together, these findings suggest complex genetic control of regeneration capacity, and justify screening efforts aimed at identifying highly responsive genotypes.

The regeneration response in spinach also varies among different explant types (Komai et al., 1996) and roots were found to be superior explants in many studies (Xiao and Branchard, 1995; Komai et al., 1996; Knoll et al., 1997). Hence, we adopted a procedure described in Knoll et al. (1997), in an attempt to increase the regeneration response of the spinach cultivar 'Matador', which is a dominant cultivar in the Serbian market.

This research was initiated with the aim to select individuals with high regeneration capacities from a population of 'Matador' cultivar seedlings, to propagate the selected lines vegetatively and keep them as a source of explants for different kinds of research. However, as this work confirmed poor regeneration response and high individual variability in individual seedlings randomly chosen from a population, we decided to improve the regeneration capacity of spinach by selecting the lines with the highest regeneration capacities and subjecting them to self-pollination, to obtain lines homozygous for genes controlling high embryogenic capacity. For this purpose, we chose *in vitro* system as it offers some advantages over *ex vitro* system, including constant monitoring of embryogenic response of lines from generation to generation and selecting the lines with the highest embryogenic capacity. In addition, the shoot apex-derived plants flower and set seed in parallel, enabling shortening of the time necessary for seed production. In an *ex vitro* system plant acclimatization is required, causing the loss of plants and delaying seed set. Furthermore, seeds obtained by self-fertilisation *ex vitro* have to be introduced to *in vitro* culture for regeneration response assessment and that would cause the loss of seeds due to contamination. Another important advantage of *in vitro* approach is sex modification under *in vitro* conditions. Spinach is a dioecious plant and only 11% of the cv. "Matador" plants are monoecious and could be self-pollinated (Ćulafić and Nešković, 1980). However, under *in vitro* conditions spinach plants are prone to sex modification (Al-Khayri et al., 1992; Komai et al., 1999; Komai and Masuda, 2004) enabling originally male and female plants to self-pollinate and set seeds. As far as we know, this phenomenon was not described in spinach plants grown under green house or field conditions. Therefore, the main objective of this study was to improve regeneration capacity, reliability and stability in 'Matador' for further use in molecular breeding of this cultivar.

2. Materials and methods

2.1. Culture initiation

'Matador' cultivar spinach seeds (Seme, Belgrade, Serbia) were washed with running water and a few drops of detergent. Seeds were then immersed in 30% commercial bleach (4% NaClO) for 30 min, followed by 15% bleach for 15 min, rinsed with sterile distilled water and blotted dry on a piece of sterile filter-paper. The seeds were planted in 90 mm Petri dishes (20 seeds per dish) containing 25 mL basal plant growth regulator (PGR)-free medium for germination. Non-contaminated seedlings were picked and replanted on new Petri dishes (three seedlings per dish) containing the same medium, and grown for a few additional weeks, until the seedlings developed 4–5 leaves and the root system was well developed.

2.2. Basal medium

The basal medium contained Murashige and Skoog's (1962) mineral solution (MS), 20 g/L sucrose, 100 mg/L myo-inositol, 2 mg/L thiamine, 2 mg/L pyridoxine, 5 mg/L nicotinic acid and 2 mg/L adenine, all obtained from Sigma–Aldrich (St. Louis, MO, USA). Media were gelled with 0.7% (w/v) agar (Torlak, Belgrade, Serbia) and the pH was adjusted to 5.6 using pH-meter. Media were sterilized in an autoclave at 114 °C for 25 min.

2.3. Plant material

The following plant material was subjected to regeneration induction: 30 parental (P)-seedlings (randomly chosen from a population), 23 S_1, 23 S_2, 17 S_3 and 5 S_4-seedlings (obtained by *in vitro* self-fertilisation of P, S_1, S_2 and S_3-plants, respectively) and SE-derived plants regenerated from root sections of selected P and S lines.

Apical shoots isolated from P-seedlings were cultured in test tubes (one shoot per tube), containing 10 mL of solid basal PGR-free medium for a period of 12 weeks. Apical shoot and root sections from the same seedling were marked with the same number. Apical-shoot-derived plants self-pollinated and set seeds, which were harvested aseptically and planted on basal PGR-free medium. These seedlings were designated S_1-seedlings, and marked with the same number as the parental genotype plus an individual consecutive number. All seeds were subjected to a 2-week cold treatment at 7 °C, unless specified. Similarly, S_2-, S_3- and S_4-seedlings were obtained by self-pollination of S_1, S_2 and S_3 seedling-derived plants, respectively.

SEs regenerated from root sections were removed after recordings at the end of each subculture, and cultured on solid MS medium supplemented with 5 μM 6-furfurylaminopurine (kinetin, Kin) for rooting and multiplication. Seeds obtained by self-fertilisation of SE-derived plants were also used to induce regeneration.

2.4. Regeneration procedure and culture conditions

Regeneration induction was performed as was described by Knoll et al. (1997), with some modifications. The same procedure was used for all plant materials. Lateral roots were isolated and 1 cm long apical sections were cut off and placed on solid basal medium supplemented with 20 μM α-naphthaleneacetic acid (NAA) and 5 μM gibberellic acid (GA_3). Explants were subcultured on the same medium at 4-week intervals over a 12-week period.

All cultures were maintained under cool white fluorescent light with a photosynthetic photon flux density of approximately 70 $\mu mol\ m^{-2}\ s^{-1}$ (as measured by an LI-1400 DataLogger equipped with an LI-190SA Quantum sensor, LI-COR Biosciences) for 16 h per day at 25 ± 2 °C.

2.5. Recordings and statistical analysis

A completely randomized design was used for culture placement. For regeneration induction, 6 samples (Petri dishes) with 5 subsamples (root sections) ($n = 30$) were prepared for each line. The number of SEs was recorded with the aid of a stereomicroscope at 4-week intervals over a 12-week period.

Differences in regeneration response among P lines were tested using standard analysis of variance (ANOVA), whereas differences among the 4 groups, consisting of 4 selected P lines and their offspring, were tested using a nested ANOVA. The means were separated using Fisher's LSD *post hoc* test for $P \leq 0.05$. Finally, differences in regeneration response between selected P-lines and SE-derived plants of the same line were compared using *t*-test for dependent samples.