Improved microspore embryogenesis induction and plantlet regeneration using putrescine, cefotaxime and vancomycin in Brassica napus *L.*

Behzad Ahmadi, Mehran E. Shariatpanahi, Mehdi Aghapour Ojaghkandi & Ali Akbar Heydari

Plant Cell, Tissue and Organ Culture (PCTOC)
Journal of Plant Biotechnology

ISSN 0167-6857

Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-014-0501-9

Springer

Your article is protected by copyright and all rights are held exclusively by Springer Science +Business Media Dordrecht. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Author's personal copy

Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-014-0501-9

ORIGINAL PAPER

Improved microspore embryogenesis induction and plantlet regeneration using putrescine, cefotaxime and vancomycin in *Brassica napus* L.

Behzad Ahmadi · Mehran E. Shariatpanahi · Mehdi Aghapour Ojaghkandi · Ali Akbar Heydari

Received: 16 March 2014 / Accepted: 26 April 2014
© Springer Science+Business Media Dordrecht 2014

Abstract The effects of three periods of exposure (12, 24 and 48 h) to different levels of putrescine (0, 0.2, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}), as well as three incubation periods (24, 48 and 72 h) to different levels of cefotaxime and vancomycin (0, 50, 100, 200 and 500 mg l^{-1}) on microspore embryogenesis of rapeseed cv. 'Hyola 401' were assessed. Microspore embryogenesis was enhanced about threefold compared with untreated culture following 48 h treatment with 0.2 mg l^{-1} putrescine. Putrescine treatment at 0.5 mg l^{-1} for 48 h effectively induced root formation and increased normal plantlet regeneration by 92 % when microspore-derived embryos (MDEs) were transferred to regeneration medium. The highest embryo yield (184.2 embryos Petri $dish^{-1}$) was possible when induction medium was supplemented with 50 mg l^{-1} cefotaxime for 24 h and the highest normal regeneration was observed in cultures exposed to 50 and 100 mg l^{-1} at all durations tested. More abnormal MDEs (76 and 82 %) were observed when microspores treated with 200 and 500 mg l^{-1} cefotaxime many of which failed to regenerate normally and resulted in callusing. Vancomycin at 100 mg l^{-1} during the 48 h exposure increased the number of MDEs (181.6 embryos Petri $dish^{-1}$) in contrast to untreated cultures (93.6 embryos Petri $dish^{-1}$) but, normal plantlet regeneration decreased as vancomycin level increased and high callusing (84 and 90 %) was observed with 200 and 500 mg l^{-1} for 72 h. Microspore embryogenesis and plant regeneration could be improved by putrescine, cefotaxime and vancomycin when appropriate levels and durations of incubation were selected.

Keywords *Brassica napus* L. · Cefotaxime · Microspore embryogenesis · Putrescine · Vancomycin

Abbreviations

ADC	*Arginine decarboxylase*
DH	Doubled haploid
ELS	Embryo-like structure
GA_3	Gibberellic acid
MDE	Microspore-derived embryo
ODC	*Ornithine decarboxylase*

Introduction

Isolated microspore culture technique is likely to remain a prominent method in *Brassica* breeding programs as it allows for rapid production of haploid or doubled haploid (DH) plants, construct genetic maps, locate genes of agronomic and economic importance, identify markers for trait selection, accelerate crop improvement programs and increase plant breeding efficiency (Forster and Thomas 2005; Chan 2010; Ferrie and Caswell 2011). Moreover, this technique is useful in gene transformation, mutation and selection, biochemical and physiological studies (Liu et al. 2005; Brew-Appiah et al. 2013; Żur et al. 2014). However, all these applications depend heavily on the efficiency of microspore embryogenesis.

B. Ahmadi · M. E. Shariatpanahi (✉) · M. A. Ojaghkandi · A. A. Heydari
Department of Tissue Culture and Gene Transformation, Agricultural Biotechnology Research Institute of Iran (ABRII), Mahdasht Road, P. O. Box 31535-1879, Karaj, Iran
e-mail: mehran.shariatpanahi@abrii.ac.ir; m_shariatpanahi2002@yahoo.com

B. Ahmadi
Department of Agronomy and Plant Breeding, University of Mohaghegh Ardabili, Ardabil, Iran

Published online: 06 May 2014

Author's personal copy

Microspore embryogenesis consists of stress-inducible reprogramming of immature pollen grains or their precursors, the microspores, diverting them from gametophytic pathway towards embryogenesis (Touraev et al. 1997; Shariatpanahi et al. 2006; Seguí-Simarro and Nuez 2008). Stress represses normal gametophytic pathway of microspores to fertile pollen and leads to intermediate stage of dedifferentiation and cell totipotency. This transitional stage allows microspores to divide and develop into embryos and regenerate complete plants (Shariatpanahi et al. 2006; Muñoz-Amatriaín et al. 2009). Various stresses are used to induce microspore embryogenesis, although the type, duration and time of application vary with the species. In the genus *Brassica*, microspores are usually inducted by osmotic stress (Ferrie and Keller 2007), heat shock and its duration (Prem et al. 2005; Ahmadi et al. 2012a), mutagenic agents (Ahmadi et al. 2012b), antioxidants (Hoseini et al. 2014) or stress hormones (Ahmadi et al. 2014).

Plants initiate a plethora of biochemical alterations i.e. up-regulation of polyamine biosynthesis, in response to the inductive stresses (Alcázar et al. 2010). Polyamines, small polycationic compounds of low molecular weight, are present in all living organisms and include spermidine, spermine, and their obligate precursor putrescine. They play important roles in a wide range of plant physiological processes such as morphogenesis, flower differentiation and initiation, regulation of rhyzogenesis, pollen viability, senescence, and response to biotic and abiotic stresses (Martin-Tanguy 2001; Alcázar et al. 2010). Polyamines are also involved in somatic embryogenesis, however, the exact mechanisms through which they exert an effect have not yet been fully elucidated (Bertoldi et al. 2004; Wu et al. 2009; Thiruvengadam et al. 2013). Polyamines enhanced embryo and callus formation from microspores by decreasing the rate of ethylene synthesis and inhibiting early senescence of cultured anthers in *Oryza sativa* (Dewi and Purwoko 2008). Promotion of somatic embryogenesis via exogenous application of polyamines or over-expression of gene(s) related to polyamine biosynthesis e.g. *ornithine decarboxylase* (*ODC*), provides convincing evidence for implication of polyamines in this process (Bastola and Minocha 1995; Kevers et al. 2000; Wu et al. 2009).

Cefotaxime is a cephalosporin antibiotic with low toxicity on eukaryotes, and effective at low doses, making it particularly attractive as a selective agent in plant tissue culture systems and gene transformation (Teixeira da Silva and Fukai 2001; Danilova and Dolgikh 2004). Besides its function in the elimination of microbial contaminations, cefotaxime exhibits auxin-like activity (Nauerby et al. 1997), enhancing effect on somatic embryogenesis and subsequently plant regeneration in *Zea mays* (Danilova and Dolgikh 2004), *Pinus pinaster* (Tereso et al. 2006), *Saccharum officinarum* L. (Mittal et al. 2009), *Solanum chacoense* (Rakosy-Tican et al. 2011), *Centella asiatica* L. (Panathula et al. 2014) and also improved microspore embryogenesis in *Triticum aestivum* L. and triticale (Asif et al. 2013). However, it inhibited somatic embryo development and adventitious bud regeneration in conifer (Sarma et al. 1995; Holland et al. 1997).

Vancomycin, an antibiotic agent classified as glycopeptides, is widely used in tissue culture systems against gram-positive bacteria. Its mode of action involves the inhibition of cell wall synthesis by binding to building blocks of peptidoglycan (monomers of *N*-acetylglucosamine and *N*-acetylmuramic acid) and preventing transpeptidase to form new blocks of cell wall, causing leakage in cellular contents of bacteria and ultimately death (Hammes and Neuhaus 1974). Being an antibiotic agent, vancomycin also promoted cell division and enhanced protoplast plating efficiency by twofold in *T. aestivum* (Simmonds and Grainger 1993). However, vancomycin inhibited embryo-like structure (ELS) formation in isolated microspore cultures of *T. aestivum* and triticale (Asif et al. 2013). In comparison with cefotaxime, information about regulatory role(s) of vancomycin in different aspects of somatic embryogenesis is rather limited.

Despite many studies on microspore embryogenesis in *Brassica napus* L., the effects of polyamines and antibiotics are not well explored. In this study, the effects of different levels and durations of exposure to putrescine, cefotaxime and vancomycin on the efficiency of microspore embryogenesis and subsequently, regeneration of MDEs were assessed in *B. napus* L. to improve microspore embryogenesis and plantlet regeneration.

Materials and methods

Donor plants and growth conditions

Brassica napus cv. 'Hyola 401' was the test plant. Donor plants were grown in a greenhouse at a day/night temperature of 15–18/7–10 °C under natural light condition during the months of September–February. Plants were irrigated three times a week.

Microspore culture

Microspore culture was carried out according to Coventry and Kott (1998) with some modifications. Buds 2.5–3.5 mm in length containing a mixed population of mid to late-uni-nucleate microspores were harvested from the main and lateral branches of donor plants that had reached anthesis after about 90–110 days. These buds were

Author's personal copy

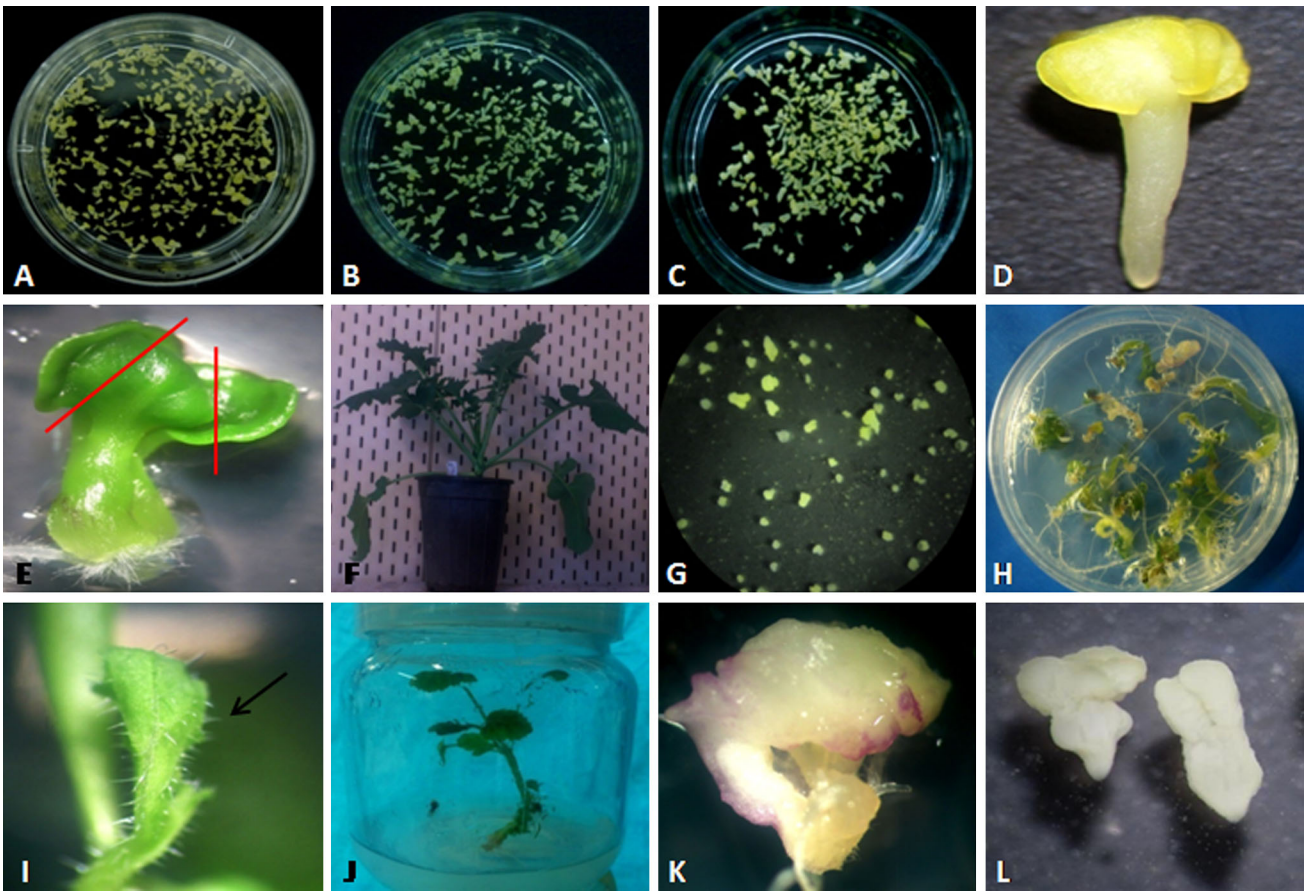

Fig. 1 *B. napus* L. cv. 'Hyola 401' microspore embryogenesis in the presence of **a** putrescine (0.2 mg l^{-1} for 48 h); **b** cefotaxime (50 mg l^{-1} for 24 h); **c** vancomycin (100 mg l^{-1} for 48 h); **d** cotyledonary MDE; **e** partial removal of cotyledons for better germination; **f** regenerated plants in the soil following gradual adaptation; **g** globular and heart-stage pro-embryos (1.0 mg l^{-1} putrescine for 48 h); **h** rooted-MDEs in B5 regeneration medium (0.5 mg l^{-1} putrescine for 48 h); **i** adventitious root formation on cotyledons (0.5 mg l^{-1} putrescine for 48 h); **j** normal plantlet regeneration; **k** callogenesis (0.5 mg l^{-1} putrescine for 48 h); **l** abnormal MDEs (200 and 500 mg l^{-1} cefotaxime)

immersed in 2.5 % sodium hypochlorite (Golrang, Tehran, Iran) with gentle shaking for 10 min followed by two 5-min washes with cold (4 °C) sterile distilled water. Approximately 130–150 sterilized buds were placed in a glass tube and gently macerated into 20 ml of liquid NLN-13 (Lichter 1982) medium supplemented with 13 % (w/v) sucrose (Duchefa Biochemie, Haarlem, The Netherlands) using a sterile glass rod. The crude suspension was filtered through a 40 µm metal mesh (Damavand Tes Sieve Ltd. Tehran, Iran), collected into two 15 ml centrifuge tubes and the volume was adjusted with fresh NLN-13 medium to 12 ml. The filtrate was centrifuged at 100×*g* for 4 min. The supernatant was decanted and the pellet was rinsed in fresh NLN-13 medium. This procedure was repeated twice. Finally the plating density was adjusted to 2×10^4 microspores ml^{-1} using a hemocytometer (Precicolor, Germany). Microspore suspension (5 ml) was dispensed into 6 cm sterile plastic Petri dishes (Farazbin, Tehran, Iran) and cultures were incubated at 30 ± 0.5 °C in the dark for 14 days. Once embryos were visible to the naked eye (Fig. 1a–c), the Petri dishes were transferred onto a rotary shaker in the dark at 55 rpm.

Putrescine, cefotaxime and vancomycin treatment

Putrescine (Sigma-Aldrich, St. Louis, MO, USA), cefotaxime and vacomycin (Duchefa Biochemie, Haarlem, The Netherlands) were dissolved in double-distilled water with gentle shaking at room temperature in the dark. The pH was adjusted at 6.0 with 1 N NaOH and 1 N HCl and maintained in a refrigerator at −20 °C until needed. After determining plating density (2×10^4 microspores ml^{-1}) and dispensing microspore suspension into the Petri dishes, different levels of filter-sterilized (0.22 µm filter) putrescine (0, 0.2, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}), cefotaxime and vancomycin (0, 50, 100, 200 and 500 mg l^{-1}) were added to the culture medium for three time periods (12, 24 and 48 h for putrescine and 24, 48 and 72 h for cefotaxime and

Author's personal copy

vancomycin) and incubated at 30 °C in the dark. Residuals were removed by centrifugation at 100×*g* for 5 min. Plating density was adjusted to 2×10^4 microspores ml^{-1} and the suspension was dispensed into the same Petri dishes and incubated at 30 °C.

MDE regeneration

Microspore-derived embryos measuring 4–6 mm in length (Fig. 1d) were transferred onto B5 medium (Gamborg et al. 1968) containing 0.1 mg l^{-1} gibberellic acid (GA_3, Fluka, Buchs, Switzerland), 2 % sucrose, pH 5.7 and 0.7 % agar (Duchefa Biochemie, Haarlem, The Netherlands) and after incubating at 4 ± 0.5 °C in the dark for 1 week, the Petri dishes were maintained at 25 ± 1 °C under a 16-h photoperiod with light intensity of 40 µE m^{-2} s^{-1} for 1 week. Subsequently, the distal half of the cotyledons was sliced off (Fig. 1e) and the MDEs were placed onto B5 medium containing 1 % sucrose, pH 5.7, 0.6 % agar and without GA_3 for plant development (Ahmadi et al. 2012b). Regenerated plantlets were transferred to pots containing pit and perlite and maintained in a growth chamber at 24 ± 1 °C under a 16-h photoperiod with light intensity of 150 µE m^{-2} s^{-1} for 2 weeks. Then, gradual adaptation was followed to greenhouse conditions (Fig. 1f).

Experimental design and statistical analysis

The experiments were conducted in a factorial arrangement based on a completely randomized design (CRD) to evaluate the effect of different factors. Entire experiments were repeated twice. Each treatment had five replications (Petri dishes). Data analyses were performed using SPSS software version 17 and the means were compared using Duncan's multiple range (DMRT) test at $\alpha = 0.01$ following analysis of variance and the frequencies (%) were compared using a Chi square test at $\alpha = 0.01$.

Results

Putrescine treatment

Putrescine level and duration of exposure significantly affected microspore embryogenesis (Table 1). Embryogenesis increased about threefold in cultures exposed to 0.2 mg l^{-1} putrescine for 48 h in relation to untreated cultures. However, microspore embryogenesis sharply decreased as putrescine level increased, and embryogenesis was completely inhibited in the cultures incubated for 24 and 48 h at 2.0 and 5.0 mg l^{-1} putrescine. Globular and heart-stage pro-embryos (Fig. 1g) were observed with 1.0 mg l^{-1} for 48 h all of which failed to proceed further into the fully developed MDEs. MDEs with a high frequency of rooting (Fig. 1h, 94 and 89 %, Table 2) on B5 regeneration medium and adventitious root formation on cotyledons (Fig. 1i, data not shown) were obtained with 24 and 48 h application of 0.5 mg l^{-1}. Putrescine level and duration of its application also affected the path of MDE regeneration (via normal regeneration or callogenesis) into the whole plantlet (Table 3). High normal plantlet regeneration (Fig. 1j) was observed in cultures exposed to 0.2 and 0.5 mg l^{-1} at all durations tested and higher levels resulted in higher callusing (Fig. 1k).

Table 1 Mean number of embryos formed Petri $dish^{-1}$ for various levels of putrescine and duration of incubation in *B. napus* L. cv. 'Hyola 401'

Putrescine level (mg l^{-1})	Duration of putrescine treatment		
	12 h	24 h	48 h
0	75.2 ± 9.2 bc*	80.4 ± 15.4 c	84.6 ± 13.3 c
0.2	101.4 ± 13.8 b	146.0 ± 13.2 b	270.2 ± 15.6 a
0.5	148.6 ± 17.4 a	218.4 ± 16.8 a	168.8 ± 13.9 b
1.0	89.0 ± 13.2 bc	96.0 ± 11.5 c	49.0 ± 10.9 d
2.0	62.0 ± 11.3 cd	41.8 ± 9.7 d	0 e
5.0	35.2 ± 8.8 d	0 e	0 e

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT ($P = 0.01$)

Table 2 Effect of various levels of putrescine and duration of incubation on frequency (%) of rooted MDEs on the regeneration medium in *B. napus* L. cv. 'Hyola 401' (for each treatment, 100 MDEs were randomly counted)

Putrescine level (mg l^{-1})	Duration of putrescine treatment		
	12 h	24 h	48 h
0	32*	41*	37*
0.2	64	59	67
0.5	75	94	89
1.0	53	61	–
2.0	44	56	–
5.0	38	–	–

* Within the column, significant differences were found according to the Chi squared test ($P = 0.01$)

Cefotaxime treatment

The highest embryo yield (184.2 embryos Petri $dish^{-1}$) was possible when the induction medium was supplemented with 50 mg l^{-1} cefotaxime for 24 h (Table 4). However, cefotaxime was not advantageous on microspore embryogenesis at longer durations. There was a sharp decrease in microspore embryogenesis as cefotaxime was increased,

Author's personal copy

Table 3 Effect of various levels of putrescine and duration of incubation on two paths of MDE regeneration (normal regeneration and callogenesis) in *B. napus* L. cv. 'Hyola 401' (for each treatment, 100 MDEs were randomly counted)

Duration of putrescine treatment	Putrescine level (mg l^{-1})	Type of MDE regeneration (%)	
		Normal regeneration	Callogenesis
12 h	0	41*	59*
	0.2	56	44
	0.5	68	32
	1.0	29	71
	2.0	12	88
	5.0	0	100
24 h	0	38	62
	0.2	64	36
	0.5	73	27
	1.0	15	85
	2.0	0	100
	5.0	–	–
48 h	0	47	53
	0.2	83	17
	0.5	92	8
	1.0	–	–
	2.0	–	–
	5.0	–	–

* Within the column, significant differences were found according to the Chi squared test ($P = 0.01$)

Table 4 Mean number of embryos formed Petri $dish^{-1}$ for various levels of cefotaxime and duration of incubation in *B. napus* L. cv. 'Hyola 401'

Cefotaxime level (mg l^{-1})	Duration of cefotaxime treatment		
	24 h	48 h	72 h
0	78.0 ± 13.6 b*	89.2 ± 11.9 b	82.8 ± 14.2 a
50	184.2 ± 16.3 a	127.0 ± 10.3 a	69.6 ± 12.0 a
100	98.4 ± 11.0 b	78.4 ± 13.6 b	21.0 ± 9.4 bc
200	73.6 ± 14.3 b	67.8 ± 11.5 b	33.8 ± 12.6 b
500	23.8 ± 9.7 c	0 c	0 c

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT ($P = 0.01$)

and 500 mg l^{-1} cefotaxime completely inhibited microspore embryogenesis in cultures incubated for 48 and 72 h. In addition, higher percentage of abnormal MDEs (Fig. 1l) were observed in cultures exposed to 200 and 500 mg l^{-1} cefotaxime (Table 5), many of which failed to regenerate normally and resulted in callusing. Cefotaxime level and duration of its application also affected the recovery of MDEs into the normal plantlets (Table 6). Higher normal

Table 5 Effect of various levels of cefotaxime and duration of incubation on frequency (%) of abnormal MDE formation in *B. napus* L. cv. 'Hyola 401' (for each treatment, 100 MDEs were randomly counted)

Cefotaxime level (mg l^{-1})	Duration of cefotaxime treatment		
	24 h	48 h	72 h
0	26*	19*	22*
50	28	34	42
100	35	38	45
200	64	68	76
500	82	–	–

* Within the column, significant differences were found according to the Chi squared test ($P = 0.01$)

Table 6 Effect of various levels of cefotaxime and duration of incubation on two paths of MDE regeneration (normal regeneration and callogenesis) in *B. napus* L. cv. 'Hyola 401' (for each treatment, 100 MDEs were randomly counted)

Duration of cefotaxime treatment	Cefotaxime level (mg l^{-1})	Type of MDE regeneration (%)	
		Normal regeneration	Callogenesis
24 h	0	37*	63*
	50	69	31
	100	74	26
	200	28	72
	500	7	93
48 h	0	34	66
	50	83	17
	100	77	23
	200	13	87
	500	–	–
72 h	0	41	59
	50	64	36
	100	71	29
	200	6	94
	500	–	–

* Within the column, significant differences were found according to the Chi squared test ($P = 0.01$)

regeneration was obtained with 50 and 100 mg l^{-1} at all durations tested.

Vancomycin treatment

Vancomycin (100 mg l^{-1}) during the 48 h exposure caused a significant increase in the number of MDEs generated (Table 7). Vancomycin at 50 mg l^{-1} was not effective on microspore embryogenesis in contrast to the control at all durations. In addition, there was no significant difference

Author's personal copy

Table 7 Mean number of embryos formed Petri dish^{-1} for various levels of vancomycin and duration of incubation in *B. napus* L. cv. 'Hyola 401'

Vancomycin level (mg l^{-1})	Duration of vancomycin treatment		
	24 h	48 h	72 h
0	88.2 ± 14.9 b*	93.6 ± 11.4 c	77.4 ± 12.1a
50	104.8 ± 12.2 b	111.2 ± 16.3 bc	97.0 ± 9.4 a
100	147.0 ± 17.4 a	181.6 ± 13.6 a	93.6 ± 11.6 a
200	109.6 ± 8.8 b	135.8 ± 9.7 b	102.2 ± 15.1 a
500	94.0 ± 12.7 b	102.4 ± 10.6 c	91.8 ± 14.4 a

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT ($P = 0.01$)

Table 8 Effect of various levels of vancomycin and duration of incubation on two paths of MDE regeneration (normal regeneration and callogenesis) in *B. napus* L. cv. 'Hyola 401' (for each treatment, 100 MDEs were randomly counted)

Duration of cefotaxime treatment	Cefotaxime level (mg l^{-1})	Type of MDE regeneration (%)	
		Normal regeneration	Callogenesis
24 h	0	35*	65*
	50	38	62
	100	47	53
	200	27	73
	500	22	78
48 h	0	43	57
	50	44	56
	100	41	59
	200	31	69
	500	18	82
72 h	0	40	60
	50	46	54
	100	37	63
	200	16	84
	500	10	90

* Within the column, significant differences were found according to the Chi squared test ($P = 0.01$)

between vancomycin levels and untreated cultures with 72 h incubation. Unlike cefotaxime, there was no inhibitory effect of high concentrations of vancomycin on microspore embryogenesis at longer durations. Normal plantlet regeneration decreased as vancomycin level was increased and high callogenesis (84 and 90 %) was observed with 200 and 500 mg l^{-1} vancomycin for 72 h (Table 8).

Discussion

Putrescine is produced by plants in response to a number of stresses, including some that are used to induce microspore embryogenesis. Putrescine level and duration of its application significantly affected microspore embryogenesis and 0.2 mg l^{-1} for 48 h had the best effect on embryogenesis (270.2 embryos Petri dish^{-1}). Working on somatic embryogenesis in *Gossypium hirsutum* L., Sakhanokho et al. (2005) noted a 53-fold increase in embryogenesis induction when 0.5 mg l^{-1} putrescine was added to culture medium. Urano et al. (2005) isolated T-DNA insertional mutants for two *arginine decarboxylase* (*ADC*) genes in *Arabidopsis*, namely *ADC1* and *ADC2*, which are involved in putrescine biosynthesis from the amino acid arginine. Lethal defect on somatic embryogenesis was observed after crossing and generating double mutation on *ADC1* and *ADC2*. Increase in free polyamines and their biosynthetic enzymes are associated with rapid cell division in many plant systems, e.g. somatic embryogenesis (Kaur-Sawhney et al. 2003). However, according to our results, high levels of putrescine were detrimental to MDE production, so that 1.0 mg l^{-1} prevented MDE development from pro-embryo structures and microspore embryogenesis completely inhibited with 2.0 and 5.0 mg l^{-1} in the cultures incubated for 24 and 48 h. Thiruvengadam et al. (2013) noted a sixfold increase in the case of free putrescine in embryogenic calli of *Momordica dioica*, but, total free putrescine declined as embryos began to develop. Cells undergo division when containing high levels of free polyamines and undergo expansion and development when containing low levels of free polyamines (Kaur-Sawhney et al. 2003).

When MDEs are placed on regeneration medium, the cotyledons turn green, a primary root develops, but the plumule rarely produces a shoot and thus many MDEs do not regenerate normally and develop into an irregular mass of tissue i.e. callogenesis (Babbar et al. 2004; Ahmadi et al. 2014). Low conversion of MDEs into the normal plantlets can constitute a serious hurdle as it reduces the likelihood of recovering transformation events. Thus, haploid plant regeneration without an intervening callus phase is highly desirable to apply this technique to plant breeding. The path of MDE regeneration into the whole plantlet was affected by putrescine treatment. High normal plantlet

Author's personal copy

regeneration was achieved using 0.2 and 0.5 mg l^{-1} at all durations tested. According to Sakhanokho et al. (2005), putrescine (0.5 mg l^{-1}) promoted the conversion of somatic embryos of *G. hirsutum* into the normal plantlets. However, according to our results, higher concentrations and longer durations resulted in higher callogenesis. Working on somatic embryogenesis in *Lasiurus scindicus*, *Sorghum halepense* and *Urochloa panicoides*, Kackar and Shekhawat (2007) reported a substantial increase in putrescine biosynthesis in the calli which lost their embryogenic competence, suggesting that higher putrescine content is associated with higher callusing.

Cefotaxime (50 mg l^{-1} for 24 h) could improve microspore embryogenesis in contrast to untreated cultures. According to Asif et al. (2013), induction medium supplemented with cefotaxime (50 and 100 mg l^{-1}) substantially increased ELS formation and green plant regeneration from microspores of *T. aestivum* and triticale. Cefotaxime and its breakdown products interfere with ethylene biosynthesis and also have auxin-like activity (Pius et al. 1993; Nauerby et al. 1997; Danilova and Dolgikh 2004). Microspore embryogenesis is strongly regulated by exogenously applied and differential distribution of the plant hormone, auxin (Zhao and Sun 2005; Ardebili et al. 2011; Dubas et al. 2014). However, according to our results, cefotaxime had a strong inhibitory effect on MDE formation, in which, 500 mg l^{-1} for 48 and 72 h completely inhibited microspore embryogenesis. The inhibitory effect of high levels of cefatoxime (400 mg l^{-1}) was also observed in somatic embryogenesis of *P. pinaster* (Tereso et al. 2006).

Higher normal regeneration was achieved using 50 and 100 mg l^{-1} cefotaxime at all durations tested. According to Yu and Wei (2007), 100 mg l^{-1} cefotaxime was the optimum concentration for normal regeneration capacity of mature embryos in *T. aestivum* and also regenerated plantlets showed more vigorous growth compared with untreated cultures. Working on two cultivars of *Catharanthus roseus* (namely Pacific Coral and Sunstorm Rose), Swanberg and Dai (2008) noted that cefotaxime at 250 mg l^{-1} increased normal shoot regeneration rate from 44 % to 54.5 % in cv. Pacific Coral, but slightly decreased shoot regeneration from 29 % to 20 % in cv. Sunstorm Rose. According to our results, 200 and 500 mg l^{-1} cefotaxime used for 48 and 72 h produced high abnormal MDEs many of which failed to regenerate normally and resulted in callusing. Stimulatory or inhibitory effect of cefotaxime on regeneration capacity varies greatly with the type of explant, species, and even among different genotypes of the same species (Bhau and Wakhlu 2001).

Microspore embryogenesis was also affected by vancomycin levels and duration of its application and the highest microspore embryogenesis (181.6 embryos Petri dish^{-1}) was achieved using 100 mg l^{-1} for 48 h. Information about possible regulatory role(s) of vancomycin on somatic embryogenesis induction is limited. Asif et al. (2013) noted that vancomycin at 100 and 500 mg l^{-1} significantly reduced ELS formation and the frequency of albino plants in isolated microspore culture of *T. aestivum*. Our results revealed that, vancomycin is not effective on microspore embryogenesis at low doses (50 mg l^{-1}) and at long durations (72 h). Also, normal plantlet regeneration decreased as vancomycin level was increased. According to Takasaki et al. (1996), high dose of vancomycin (750 mg l^{-1}) greatly reduced shoot regeneration from hypocotyls explants in eight cultivars of *B. rapa*. However, positive effect of vancomycin on normal plantlet regeneration was observed when used alone or in combination with other antibiotics. Burgos and Alburquerque (2003) noted that medium containing vancomycin (200 mg l^{-1}) caused a 204 % improvement of plantlet regeneration from apricot (*Prunus armeniaca* L.) leaves when used alone and a 98 % improvement in combination with cefotaxime. However, according to our results, higher levels of vancomycin (200 and 500 mg l^{-1}) resulted in higher callusing at all durations tested.

Acknowledgments This research was supported by grants from Agricultural Biotechnology Research Institute of Iran (ABRII) Project No. 1-05-05-8601.

References

Ahmadi B, Ghadimzadeh M, Moghaddam AF, Alizadeh K, Teixeira da Silva JA (2012a) Bud length, plating density, and incubation time on microspore embryogenesis in *Brassica napus*. Int J Veg Sci 18:346–357

Ahmadi B, Alizadeh K, Teixeira da Silva JA (2012b) Enhanced regeneration of haploid plantlets from microspores of *Brassica napus* L. using bleomycin, PCIB, and phytohormones. Plant Cell Tissue Organ Cult 109:525–533

Ahmadi B, Shariatpanahi ME, Teixeira da Silva JA (2014) Efficient induction of microspore embryogenesis using abscisic acid, jasmonic acid and salicylic acid in *Brassica napus* L. Plant Cell Tissue Organ Cult 116:343–351

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 231:1237–1249

Ardebili SH, Shariatpanahi ME, Amiri R, Emamifar M, Nematzadeh G, Noori SAS, Oroojloo M, Heberle-Bors E (2011) Effect of 2,4-D as a novel inducer of embryogenesis in microspores of *Brassica napus* L. Czech J Genet Plant Breed 47:114–122

Asif M, Eudes F, Randhawa H, Amundsen E, Yanke J, Spaner D (2013) Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale. Plant Cell Rep 32:1637–1646

Babbar BS, Agarwal PK, Sahay S, Bhojwani SS (2004) Isolated microspore culture of *Brassica*: an experimental tool for developmental studies and crop improvement. Indian J Biotechnol 3:185–202

Author's personal copy

Bastola DR, Minocha SC (1995) Increased putrescine biosynthesis through transfer of mouse ornithine decarboxylase cDNA in carrot promotes somatic embryogenesis. Plant Physiol 109:63–71

Bertoldi D, Tassoni A, Martinelli L, Bangi N (2004) Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars. Physiol Plant 120:657–666

Bhau BS, Wakhlu AK (2001) Effect of some antibiotics on the in vitro morphogenetic response from callus cultures of *Coryphantha elephantidens*. Biol Plant 4(1):19–24

Brew-Appiah RAT, Ankrah N, Liu W, Konzak CF, von Wettstein D, Rustgi A (2013) Generation of doubled haploid transgenic wheat lines by microspore transformation. PLoS One. doi:10.1371/journal.pone.0080155

Burgos L, Alburquerque N (2003) Ethylene inhibitors and low kanamycin concentrations improve adventitious regeneration from apricot leaves. Plant Cell Rep 21:1167–1174

Chan SWL (2010) Chromosome engineering: power tools for plant genetics. Trends Biotechnol 28:650–710

Coventry J, Kott LS (1998) Doubled haploid technology for spring and winter *Brassica napus*, revised ed. OAC Publication, University of Guelph, Ontario, Canada. Technol Bull, p 42

Danilova SA, Dolgikh YI (2004) The stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture. Russ J Plant Physiol 51(4):559–562

Dewi IS, Purwoko BS (2008) Role of polyamines in inhibition of ethylene biosynthesis and their effects on rice anther culture development. Indones J Agric Sci 9(2):60–67

Dubas E, Moravčíková J, Libantová J, Matušíková I, Benková E, Żur I, Krzewska M (2014) The influence of heat stress on auxin distribution in transgenic *B. napus* microspores and microspore-derived embryos. Protoplasma. doi:10.1007/s00709-014-0616-1

Ferrie AMR, Caswell KL (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104:301–309

Ferrie AMR, Keller WA (2007) Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in *Brassicaceae*. In Vitro Cell Dev Biol Plant 43:348–355

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. In: Janis J (ed) Plant Breed Rev. Wiley, New York 25:57–88

Gamborg OL, Miller RA, Ojima L (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hammes WP, Neuhaus FC (1974) Mechanism of action of vancomycin—inhibition of peptidoglycan synthesis in *Gaffkya homari*. Antimicrob Agents Chemother 6:722–728

Holland L, Gemmell JE, Charity JA, Walter C (1997) Foreign gene transfer into *Pinus radiate* cotyledons by *Agrobacterium tomefaciens*. N Z J For Sci 27:289–304

Hoseini M, Ghadimzadeh M, Ahmadi B, Teixeira da Silva JA (2014) Effects of ascorbic acid, alpha-tocopherol, and glutathione on microspore embryogenesis in *Brassica napus* L. In Vitro Cell Dev Biol Plant. doi:10.1007/s11627-013-9579-8

Kackar A, Shekhawat NS (2007) Plant regeneration through embryogenesis and polyamine levels in cultures of grasses of Thar Desert. J Cell Mol Biol 6(2):121–127

Kaur-Sawhney R, Tiburcio AF, Altabella T, Galston AW (2003) Polyamines in plants: an overview. J Cell Mol Biol 2:1–12

Kevers C, Gal NL, Monteiro M, Dommes J, Gaspar T (2000) Somatic embryogenesis for *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathway. Plant Growth Regul 31:209–214

Lichter R (1982) Induction of haploid plants from isolated pollens of *Brassica napus*. Z Pflanzenphysiol 105:427–434

Liu S, Wang H, Zhang J, Fitt BDL, Xu Z, Evans N, Liu Y, Yang W, Guo X (2005) *In vitro* mutation and selection of doubled-haploid *Brassica napus* lines with improved resistance to *Sclerotinia sclerotiorum*. Plant Cell Rep 24:133–144

Martin-Tanguy J (2001) Metabolism and function of polyamines in plants: recent development (new approaches). Plant Growth Regul 34:135–148

Mittal P, Gosal SS, Senger A, Kumar P (2009) Impact of cefotaxime on somatic embryogenesis and shoot regeneration in sugarcane. Physiol Mol Biol Plants 15(3):257–265

Muñoz-Amatriaín M, Svensson JT, Castillo AM, Close TJ, Vallés MP (2009) Microspore embryogenesis: assignment of genes to embryo formation and green vs. albino plant production. Funct Integr Genomics 9:311–323

Nauerby B, Billing Z, Wyndaele R (1997) Influence of antibiotic timentin on plant regeneration compared to carbenicillin and cefatoxime in concentrations suitable for elimination of *Agrobacterium tomefaciens*. Plant Sci 123:169–177

Panathula CS, Mahadev MDN, Naidu CV (2014) The stimulatory effect of the antimicrobial agents bavistin, cefotaxime and kanamycin on in vitro plant regeneration of *Centella asiatica* (L.)—an important antijaundice medicinal plant. Am J Plant Sci 5:279–285

Pius J, George L, Eapen S, Rao PS (1993) Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and cefotaxime. Plant Cell Tissue Organ Cult 32:91–96

Prem D, Gupta K, Agnihotri A (2005) Effect of various exogenous and endogenous factors on microspore embryogenesis in Indian mustard (*Brassica juncea* L. Czern and Coss). In Vitro Cell Dev Biol Plant 41:266–273

Rakosy-Tican E, Aurori CM, Aurori A (2011) The effects of cefotaxime and silver thiosulphate on in vitro culture of *Solanum chacoense*. Rom Biotechnol Lett 16(4):6369–6377

Sakhanokho HF, Ozias-Akins P, May OL, Chee PW (2005) Putrescine enhances somatic embryogenesis and plant regeneration in upland cotton. Plant cell Tissue Organ Cult 81:91–95

Sarma KS, Evans NE, Selby C (1995) Effect of carbenicillin and cefotaxime on somatic embryogenesis of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). J Exp Bot 46:1779–1781

Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134:1–12

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiol Plant 127:519–534

Simmonds JA, Grainger JL (1993) The toxicity of antibiotics to protoplast cultures of *Triticum aestivum* L. Plant Sci 89:209–214

Swanberg A, Dai W (2008) Plant regeneration of periwinkle (*Catharanthus roseus*) via organogenesis. Hortic Sci 43(3):832–836

Takasaki T, Hatakeyama K, Ojima K, Watanabe M, Toriyama K, Hinata K (1996) Effects of various factors (hormone combinations, genotypes and antibiotics) on shoot regeneration from cotyledon explants in *Brassica rapa* L. Plant Tissue Cult Lett 13(2):177–180

Teixeira da Silva JA, Fukai S (2001) The impact of carbencillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. J Appl Hortic 3(1):3–12

Tereso S, Miguel C, Maroco J, Oliveira MM (2006) Susceptibility of embryogenic and organogenic tissues of maritime pine (*Pinus pinaster*) to antibiotics used in *Agrobacterium*-mediated genetic transformation. Plant Cell Tissue Organ Cult 87:33–40

Author's personal copy

Thiruvengadam M, Rekha KT, Jayabalan N, Praveen N, Kim EH, Chung IM (2013) Effect of exogenous polyamines enhances somatic embryogenesis via suspension cultures of spine guard (*Momordica dioidca* Roxb. ex. Wild). Australian. J Crop Sci 7(3):446–453

Touraev A, Vicente O, Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends Plant Sci 2:297–302

Urano K, Hobo T, Shinozaki K (2005) *Arabidopsis ADC* genes involved in polyamine biosynthesis are essential for seed development. FEBS Lett 579:1557–1564

Wu X-B, Wang J, Liu J-H, Deng X-X (2009) Involvement of polyamine biosynthesis in somatic embryogenesis of Valencia sweet orange (*Citrus sinensis*) induced by glycerol. J Plant Physiol 166:52–62

Yu Y, Wei ZM (2007) Influences of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. Biol Plant 52(3):553–556

Zhao J, Sun MX (2005) Influence of exogenous auxin on microspore embryogenesis in tobacco. Wuhan Bot Res 23(6):519–523

Żur I, Dubas E, Krzewska M, Sanchez-Díaz RA, Castillo AM, Vallés MP (2014) Changes in gene expression patterns associated with microspore embryogenesis in hexaploid triticale (× *Triticosecale* Wittm.). Plant Cell Tissue Organ Cult 116:261–267