ORIGINAL ARTICLE

Somatic embryogenesis and shoot organogenesis from the hypocotyl slices and free radical scavenging activity of regenerants of collard greens (*Brassica oleracea* L. var. *acephala*)

Nevena Banjac[1] · Branka Vinterhalter[1] · Dijana Krstić-Milošević[1] · Jelena Milojević[1] · Ljiljana Tubić[1] ·
Nabil Ghalawenji[1] · Snežana Zdravković-Korać[1]

Abstract

Brassica oleracea var. *acephala* is an important leafy vegetable that has been widely consumed as a high-nutrient, low-calorie food. Because of the plant's biennial and self-incompatibility nature, biotechnological approaches are alternative way for propagation and breeding improvements. Since tissue culture studies have been focused in other *B. oleracea* representatives, the aim of the present study was to achieve effective regeneration protocol distinctive for collard greens, and evaluate the total phenolic content and antioxidant activity of regenerants. The effect of 3 cytokinins [thidiazuron (TDZ), 6-benzyladenine (BA) and 6-furfuryladenine (kinetin, KIN)] at increasing concentrations (0, 5, 7.5, 10, 20 or 30 μM) in combination with tenfold lower concentration of 1-naphtaleneacetic acid (NAA) (0, 0.5, 0.75, 1, 2 or 3 μM, respectively) on the regeneration from hypocotyl slices was studied. Histological analysis revealed the two regeneration pathways, somatic embryogenesis and shoot organogenesis, simultaneously occurred in the same explant, regardless of the cytokinin/NAA combinations used. The regeneration frequency of 95.9%, with 7.5 morphogenic structures regenerated per explant, and the healthy appearance of regenerated plants indicated the optimal combination 20 μM TDZ + 2 μM NAA. TDZ at 5 μM provided the high somatic embryo proliferation rate by generation of secondary embryos (7.79) along with the lowest rate of their abnormalities. Embryo-developed plants were successfully acclimatised (above 90%). The plants regenerated and proliferated on TDZ-containing media had higher total phenolic content that correlated with the highest free radical scavenging activity ($IC_{50} = 19.09$ μg ml^{-1}).

Key message

Somatic embryogenesis and shoot organogenesis from hypocotyl slices of the collard greens were established. The combination 20 μM TDZ + 2 μM NAA was optimal for regeneration providing higher total phenolic content.

✉ Nevena Banjac
mitic.nevena@ibiss.bg.ac.rs

[1] Institute for Biological Research "Siniša Stanković", University of Belgrade, Despot Stefan Boulevard 142, 11060 Belgrade, Serbia

Introduction

Brassica oleracea L. var. *acephala*, collard greens, is one of the important *B. oleracea* vegetables, classified in the Acephala group, together with genetically related kale and spring greens. It is a descendant of the wild cabbage, a plant species that has originated in Asia Minor and has been consumed as food since prehistoric times. Cruciferous vegetables contain a variety of biologically active molecules and nutritional components that provide numerous health benefits (Manchali et al. 2012) and represent the most commonly

vegetables consumed worldwide. Since collard greens do not form heads, their leaves are fully exposed to sunlight that is favorably reflected on the synthesis of the phenolic compounds and anti-oxidant capacity (Ferreres et al. 2006; Ghasemzadeh et al. 2010). Collard greens are one of the most nutritious Brassica vegetables and excellent source of vitamins, minerals and insoluble dietary fibers. Nutritious collard leaves are very low in calories (providing only 30 calories per 100 g) and significantly higher in potassium content than the other leafy green tested kale, canola and cabbage (Miller-Cebert et al. 2009). Bile acid binding values, related to the cholesterol-lowering potential of food, were significantly higher for collard greens than those for the other cooked vegetable tested, including kale, mustard greens, broccoli, spinach, Brussels sprouts and green bell pepper (Kahlon et al. 2008). The main health benefits from collard greens are the cancer and osteoporosis preventive properties (Gong et al. 2006; Pereira et al. 2006). Since the US Department of Agriculture (USDA) Food and Nutrition Information Center, through the Food Guide Pyramid in 2006 (http://www.mypyramid.gov) has been recommended the consumption of dark leafy and low-fat vegetables, including collard greens, their recent rise in popularity has reinvigorated interest from both fresh and processed markets across the U.S. Data from the 2012 Census of Agriculture estimates that approximately 12,542 acres of collard greens were harvested for U.S. (USDA 2012) and market trends suggest a continued rise in acreage dedicated to their production (NYSVegExpo 2017).

Current market expansion makes the production of collard greens has received more attention by breeders and seed companies. Although numerous open-pollinated and hybrid collard cultivars are available (Olson and Freeman 2008), mainly through seed saving organizations or small seed companies, their production and quality remain affected by a variety of plant pests and diseases, including molds (fungi), bacteria, viruses and nematodes. Damage can range from minor spotting on leaves to complete loss of a crop, depending on the pathogen involved. Leaves are the marketable product in collards production; therefore, even a minor leaf spotting can result in a significant yield loss, while severe disease development can reduce quality, to the point where the crop is unmarketable. Conventional breeding programs, which would incorporate disease resistance and other desirable traits in collard greens, are difficult to carry out, because of the plant's biennial and self-incompatible nature, as well as requirements for isolation barriers in order to prevent unwanted spontaneous hybridization due to pronounced outcrossing potential of Brassica species with other cultivars and even closely related species and genera. Biotechnological approaches are some of the alternative ways to overcome these limitations and many breeders have tried to improve

Brassica vegetables by combining biotechnological with classical breeding methods. Thus, in the last two decades, considerable progress in the field of plant tissue culture and genetic transformation were achieved, particularly in cabbage (*B. olaracea* var *capitata*) (reviewed by Gerszberg 2018). Genetic engineering has been used to create *Bacillus thuringiensis* (Bt)-transgenic collard greens lines that have the potential to be used either for direct pest control, or as a ''dead end'' trap crop for Lepidoptera (Cao et al. 2005). Biotechnology was also the option of choice for the production of recombinant antigens in collard greens (Pogrebnyak et al. 2006). Since cabbages contain flavonoids, phenolic compounds with health-promoting effects, development of genetically modified plants with enhanced production of flavonoids represents the challenge of biotechnology researches in this plant group (Lännenpää 2014). Formerly, the majority of the reports of somatic embryogenesis and shoot regeneration from different explants, including hypocotyls, petioles, cotyledons, microspores etc. refer to the other members of *Brassica oleracea* (Acephala Group), primarily ornamental (Lillo and Olsen 1989; Xiushu et al. 2009; Dai et al. 2009; Wang et al. 2011; Taghizadeh and Solgi 2015; Niu et al. 2015) and sporadically edible kale (Feng et al. 2009). At the same time, no regeneration protocol distinctive for collard greens has been reported. However, widespread application of biotechnology in collard breeding programs requires further improvements of in vitro regeneration procedures, as this species has been considered recalcitrant to genetic transformation (Puddephat et al. 1996). Cao et al. (2005) obtained transformed collard plants via organogenesis, using protocols that have been developed for regeneration of other *B. oleracea* varieties (Cao and Earle 2003). Since the regeneration response was mostly influenced by genotype and considering that substantial variations among the same species, or even cultivar, are feasible, further efforts have to be introduced for optimization of collard greens' in vitro regeneration, especially in developing a procedure for somatic embryo induction. Somatic embryogenesis is a desirable and fast method of plant regeneration, useful for artificial seed production (Qamar et al. 2014), cryopreservation (Al Shamari 2014), as well as large-scale propagation and genetic manipulations (Deo et al. 2010).

In the present study, a simple, one-step regeneration protocol via both somatic embryogenesis and organogenesis was reported for collard greens. The effectiveness of thidiazuron (TDZ), 6-benzyladenine (BA) and 6-furfuryladenine (kinetin, KIN) on the morphogenic structures regeneration and proliferation capacity was examined. Although the use of these in vitro regeneration procedures could provide many benefits, cytokinins applied during regeneration process may change the chemical composition of regenerants, affecting

production of phenolic compounds too (Aremu et al. 2013; Macalalad et al. 2016). Therefore, total phenolic content, free radical scavenging activity, as well as HPLC profile of flavonoids of acclimatised plants, were studied.

Materials and methods

Plant material

Mature seeds of collard greens (*Brassica oleracea* L. var. *acephala*) were collected in 2010–2011 from an open-pollinated plant population, grown in Trebinje, Bosnia and Herzegovina, 19 km away from the Adriatic Sea (275 m altitude, latitude 42° 42′ 18″N, longitude 18° 19′ 18″E). The seeds were surface sterilized using 70% ethanol for 1 min, followed by 20% commercial NaOCl bleach (4% active chlorine), supplemented by few drops of detergent (Fairy, Procter and Gamble), fungicide Previcur® (Bayer CropScience, Monheim, Germany) and 500 mg l^{-1} antibiotic ampicillin (Panfarma d.o.o, Belgrade, Serbia) for 2 h, rinsed 4 times with sterile distilled water and blotted dry on a piece of sterile filter paper. Surface-sterilized seeds were aseptically germinated in 90 mm Petri dishes (about 5–7 seeds per dish) on MS (Murashige and Skoog 1962) basal medium with 2% (w/v) sucrose. The medium was solidified with 0.7% (w/v) agar (Torlak Institute, Belgrade, Serbia) and the pH was adjusted to 5.6 before sterilisation in an autoclave at 114 °C (80 kPa) for 25 min.

For seed germination, ampicillin was added into MS basal medium after autoclaving. Two days later, non-contaminated seedlings were picked up and replanted into the new Petri dishes containing the same medium and were grown for additional 3 days.

Explant preparation and morphogenic structure regeneration

Cotyledons, hypocotyls and roots, excised from 5-day-old in vitro-grown seedlings, were used as explants. Cotyledons were transversely bi-sectioned; roots (2.5–3.5 cm in length) were cut into five to seven pieces, while hypocotyls (1.0–1.5 cm in length) were sliced into 9–18 cross-sections (about 1.5–2.0 mm diameter and 0.8–1.0 mm thick). The hypocotyl slices were positioned with the root-side surface bottom-down on MS media and the shoot-side surface up. All slices made from a single seedling's hypocotyl (considered as genotype) were placed in one Petri dish in order from distal to proximate cotyledons end of hypocotyl. The explants were cultured on MS basal medium supplemented with 0, 5, 7.5, 10, 20 or 30 μM thidiazuron (TDZ), 6-benzyladenine (BA) or 6-furfuryladenine (kinetin, KIN) in

combination with a tenfold lower concentration (0, 0.5, 0.75, 1, 2 and 3 μM, respectively) of 1-naphthalene acetic acid (NAA) (regeneration medium). All plant growth regulators (PGRs) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The medium pH was adjusted with 1N NaOH to 5.6. TDZ was filter sterilised and added into cooled medium after autoclaving at 114 °C for 25 min. Six cotyledon or 5–7 root explants were cultured per Petri dish. For regeneration experiment, each treatment comprised ten replicates (Petri dishes), each with 9–18 explants and the whole experiment was repeated three times. Dishes were sealed with Parafilm® M (Pechiney Plastic Packing, Chicago, IL, USA) and incubated in the growth room, under a 16-h photoperiod provided by cool white fluorescent tubes, with an irradiance of ≈ 50 μmol m^{-2} s^{-1} at 25 ± 2 °C.

The regeneration frequency and the number of induced morphogenic structures, somatic embryos plus adventitious shoots, were counted jointly after 3 weeks of cultivation with the aid of a stereomicroscope.

Proliferation of somatic embryos and plant development

For proliferation, individual somatic embryos (about 5 mm in length), regenerated from the same seedling on either 20 μM TDZ, BA or KIN + 2 μM NAA, were isolated and subcultured on MS proliferation medium containing 5 μM of appropriate TDZ, BA or KIN, respectively. Individual somatic embryos subcultured on PGR-free MS medium were used as a control. The experiment was performed in three replicates, each with 5 Petri-dishes containing 5–7 single embryos, n = 78–90 in total per treatment. Cultures were incubated under the same growth conditions as stated above. The number of secondary embryos was recorded after 3 weeks.

As the next step, individual secondary somatic embryos were isolated from available clusters and transferred into Petri dishes for the first 3 weeks and after that additionally for the 3 weeks into the glass jars, both containing PGR-free MS medium for growth and rooting. Three replicates, each with 9–11 single germinated secondary somatic embryos (n = 27–31 in total per treatment) were prepared.

After 6 weeks of culture, the number of roots produced per cultured embryo-derived plantlet, length of the longest root, as well as the frequency of root formation were recorded. Plants reaching 5–10 cm in length were planted in plastic containers containing 1:1 mix of soil and perlite for acclimatisation in a greenhouse. Humidity was preserved by covering the pots with transparent polyethylene bags for 1 week. The plants were watered on alternate days.

Histological investigation of somatic embryogenesis and organogenesis

The samples of the different treatments were fixed in FAA (5 ml 40% formaldehyde: 5 ml glacial acetic acid: 90 ml 70% ethanol). After dehydration in an alcohol series (70% for 60 min, 95% for 15 min, 95% for 30 min, and 100% for 15 min), the samples were cleared with xylene and subsequently embedded in paraffin wax at 58 °C. Serial 8-μm thick sections were cut using a microtome (Reichert, Vienna, Austria), stained with Delafield haematoxylin, mounted in Canada balsam, and viewed and photographed under a light Leitz DMRB photomicroscope (Leica, Wetzlar, Germany).

Total phenolic content and HPLC profile of flavonoids

The total phenolic content (TPC) was determined in acclimatised somatic embryoderived plants, which were regenerated on MS media with 20 μM TDZ, BA or KIN + 2 μM NAA and subsequently multiplied on proliferation media containing 5 μM TDZ, BA or KIN, respectively, using Folin–Ciocalteu method (Singleton and Rossi 1965) with slight modifications. Seed-derived plants were used as a control. Plant material was air-dried at room temperature and ground to fine powder using a mortar and a pestle. The obtained powder (3 g) was extracted using 60% aqueous methanol solution (50 ml) in an ultrasonic bath for 20 min. After sonication, extraction was continued by maceration for 48 h in the dark at room temperature. The extracts were filtered through quantitative filter paper and the supernatants were evaporated to dryness in a vacuum evaporator at 50 °C. The dry extracts were stored at 4 °C until use, for further chemical analysis. The dry extracts were redissolved in methanol (2.5 mg ml^{-1}). For TPC determination 500 μl of Folin–Ciocalteu reagent (previously diluted tenfold with distilled water) and 100 μl of prepared methanol extract were mixed. After 5 min, 400 μl of sodium carbonate (0.75 g ml^{-1}) was added, and the mixture was incubated at room temperature in the dark for 2 h. The absorbance of samples was measured at 765 nm using an Agilent 8453 UV–VIS spectrophotometer. The total phenolic content was calculated from the calibration curve of gallic acid, and the results were expressed as mg of gallic acid equivalent per g of dry weight of plant extract (mg GAE g^{-1} DW). Three samples were prepared and measured per treatment and the mean values were calculated.

Prior to HPLC analysis, in order to obtain flavonoid aglycones, the dry extracts (400 mg) were redissolved in methanol (1.5 ml) and hydrolyzed with 2M HCl in the water bath at 85–90 °C for 2 h. Analysis of flavonoid aglycones was carried out on an Agilent 1100 series HPLC instrument (Agilent, Waldbronn, Germany), with a DAD detector,

on a reverse phase Zorbax SB-C18 (Agilent) analytical column (250 mm × 4.6 mm i.d., 5 μm particle size). The mobile phase consisted of solvent A (1%, v/v solution of orthophosphoric acid in water) and solvent B (acetonitrile), using the gradient elution as follows: 90–75% A 0–33 min, 75–45% A 33–40 min, 45–0% A 40–45 min. The detection wavelength was set for flavonoids at 360 nm, and the flow rate was 0.8 ml min^{-1}. Flavonoid standards kaempferol and quercetin were purchased from Sigma–Aldrich Co. Quantification was performed using calibration curves in external standard method. Results were expressed as mg per g of dry weight of plant extract (mg g^{-1} DW).

Radical scavenging activity

The free radical scavenging activity of the extracts on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was carried out according to the procedure described previously (Brand-Williams et al. 1995), with some modifications. The antiradical activity was evaluated using various concentrations of each extract ranging from 0.06 to 1 mg ml^{-1}. The reaction mixture contained 500 μl of extract and 500 μl of daily prepared 150 μM DPPH solution in methanol. The resulting mixture (1 ml) was shaken vigorously and incubated for 20 min at room temperature in the dark. Thereafter, absorbance of the samples was measured spectrophotometrically at 517 nm. All tests were performed in triplicate, with Trolox as a positive control.

The percentage of inhibition was calculated using equation:

$$\% \text{ Inhibition} = \left[\left(A_0 - A_1\right)\big/ A_0\right] \times 100$$

where A_0 is absorbance of the control solution (containing only DPPH) and A_1 is the absorbance of the samples. The IC$_{50}$ values were calculated by linear regression of plots where the abscissa represented the concentration of tested samples and the ordinate the average percent of inhibition activity from three separate tests.

Data analysis

All cultures were placed in a completely randomized design. The frequency of regeneration was calculated as the percentage of explants producing morphogenic structures, somatic embryos and adventitious shoots, and the mean number of morphogenic structures per explant was determined after 3 weeks of culture on MS regeneration media using a stereomicroscope for visualization. For somatic embryo proliferation, the total number of secondary somatic embryos produced per primary somatic embryo was recorded after 3 weeks of culture.

Percentage data were subjected to angular transformation and morphogenic structures number data to square root

transformation prior to analysis, followed by inverse transformation for presentation. The data were subjected to standard analysis of variance (ANOVA), which was performed using the General Linear Model procedure in SAS 9.0 (SAS Institute Inc., Cary, NC, USA). Significant differences in the mean values were determined by Fisher's Least Significant Difference (LSD) test at a significance level of 0.05. The experiment concerning the influence of cytokinins on morphogenic structures regeneration was designed as a two-way factorial arrangement on a completely randomized design, with ten replicates per treatment/experiment. To estimate the influence of treatments on somatic embryo proliferation, rooting and total phenolic content, one-way ANOVA was used. The correlation analysis was performed employing the Pearson's Correlation Test.

Results

At the beginning of the experiment, collard greens seeds were germinated on MS PGR-free medium at the rate of 70.7%, and 10.6% of the total number of the cultured seeds were contaminated. Since no previous information on the regeneration protocols of collard greens variety have been reported, cotyledon, root sections and hypocotyl slices of in vitro-grown seedlings were initially investigated on MS regeneration media. Cotyledon and root section explants did not display any kind of regeneration response under given conditions (data not shown) and therefore were excluded from further experiments.

Induction of somatic embryogenesis and shoot organogenesis from hypocotyl slices

The hypocotyl-slice explants cultured on MS medium in the absence of PGRs failed to regenerate while in the presence of cytokinins, the hypocotyl slices started to swell and expand in size during the first few days of culture. Very poor vesicular (bubbly) callusing was observed in the central area of the cross-section, while abundant, soft and translucent callus was formed on the peripheral explant's part after 7 days of culture on regeneration media. During the first week of culture, numerous vesicles, as harbingers of regeneration, and soon after adventitious shoots, as the first visible signs of explant regeneration, filled in the central cylinder zone of slices cultured on medium with 20 μM TDZ and
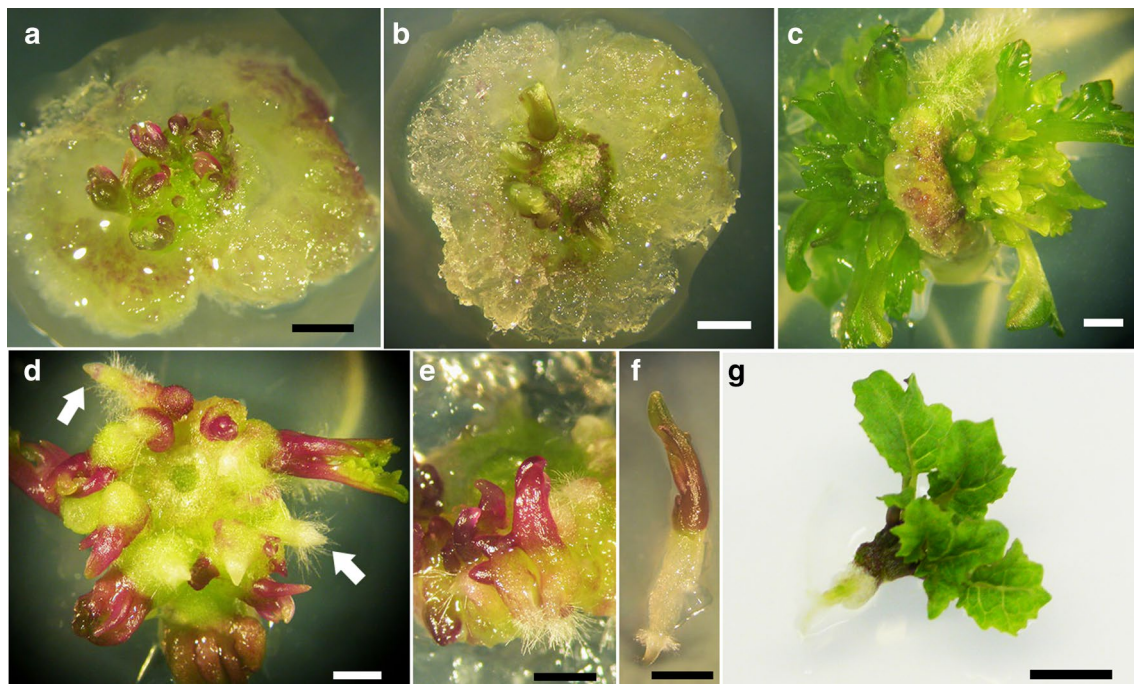

Fig. 1 Somatic embryogenesis and shoot organogenesis in *B. oleracea* var. *acephala*. (a and b) Adventitious shoots regeneration that fill (**a**), or surround (**b**) central part of the explant after 7 days of cultivation on medium with 20 μM TDZ + 2 μM NAA and 5 μM BA + 0.5 μM NAA, respectively. **c** Later phase of shoot regeneration from explants cultivated at 20 μM TDZ + 2 μM NAA medium. **d** Callus–mediated somatic embryogenesis at the periphery of the explants cultivated for 3 weeks at TDZ 20 μM + NAA 2 μM. Numerous somatic embryos were visible to arise from callus tissue by the root pole (arrows). **e** Somatic embryo's radicles elongation and somatic embryo germination into small plantlet (**f**) observed on the same regeneration medium (TDZ 20 μM + NAA 2 μM) after 3 weeks of culture. **g** The conversion of somatic embryos into whole plants after 2 weeks of culture on PGR-free MS medium. Bars = 1 mm (**a–d** and **f**), 0.5 mm (**e**) and 5 mm (**g**)

2 µM NAA (Fig. 1a), or shoots arising from cambial zone surrounding the central part of the explant after culture on medium containing 5 µM BA and 0.5 µM NAA (Fig. 1b). Adventitious shoots often appeared from shoot-side explant surface, but occasionally, both sides of hypocotyl-slice explant could regenerate after continued cultivation with 20 µM TDZ + 2 µM NAA (Fig. 1c). Explants which were cultured on medium with KIN regenerated adventitious roots in the central part, rather than adventitious shoots. If adventitious shoots were formed from the cambial region around central cylinder, as observed on explants cultured for 14 days on medium with 7.5 µM KIN + 0.75 µM NAA, then numerous adventitious roots enclosed the periphery of the explant. After two weeks of culture on primarily BA- and TDZ-containing media, somatic embryos, arising by shoot, or frequently by root pole from soft and translucent callus at the explant periphery, were observed. About one week later, soft and translucent callus became whiter and more nodular and numerous somatic embryos at different stage of development were formed on explants cultured on TDZ-containing medium (Fig. 1d). After three to four weeks of culture on medium with 20 µM TDZ + 2 µM NAA, somatic embryos reached 3–10 mm in length. In some of them, the radicles began to elongate (Fig. 1e) and somatic embryos germinated precociously into plantlets on the same regeneration medium

(Fig. 1f). The development of somatic embryos, regenerated on medium containing 20 µM TDZ + 2 µM NAA, was further continued on PGR-free MS medium, where most of the embryos germinated into plantlets with distinctly developed green shoot and root after 2 weeks (Fig. 1g) .

Histology of somatic embryogenesis and shoot organogenesis from hypocotyl slices

During the first 5 days of culture, callus tissue with large isodiametric cells was formed from the peripheral part of the explant grown on all regeneration media. As culture time was extended to 7–10 days, the first signs of morphogenesis were visible in both central area of the explant (recognizable by vascular tissue) and in the peripheral callus tissue as well. The same regeneration pathway has occurred regardless of the type of the regeneration medium used and both somatic embryogenesis and shoot organogenesis were recognized in the callus tissue of all explants. Some globular somatic embryos were observed on the surface (Fig. 2a), as well as inside of the callus tissue that formed in the peripheral part of the explant cultured for 10 days on regeneration media. At the same time, along with somatic embryos, less frequent adventitious shoots were observed in the central part of the explants (Fig. 2b). As culture duration on regeneration
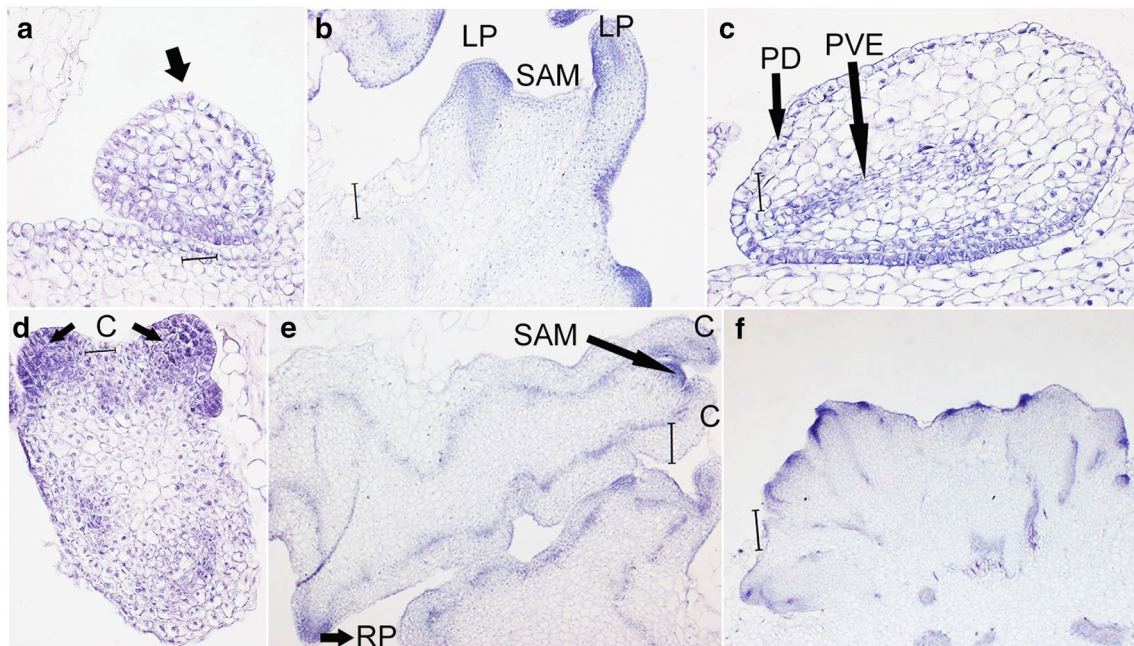

Fig. 2 Histology of somatic embryogenesis and shoot organogenesis from hypocotyl slices of *B. oleracea* var. *acephala*. **a** Globular somatic embryo formed on the surface of the callus tissue formed after 10 days at BA 20 µM + NAA 2 µM. **b** Formation of adventitious shoots with visible shoot apical meristem (SAM) and leaf primordia (LP) after 10 days of culture on medium containing TDZ 20 µM + NAA 2 µM. **c** and **d** Somatic embryos at different stages: torpedo on TDZ 20 µM + NAA 2 µM (**c**) and early-cotyledonary on KIN 20 µM + NAA 2 µM (**d**). **e** Germinating somatic embryo during culture on medium with TDZ 20 µM + NAA 2 µM. **f** Fasciated somatic embryos after 14 days of culture on regeneration medium containing BA 20 µM + NAA 2 µM. Bars = 40 µm (**a**), 100 µm (**b**), 50 µm (**c** and **d**), 160 µm (**e**) and 320 µm (**f**). *PD* Protoderm, *PVE* provascular elements, *C* cotyledon, *RP* root pole

medium was prolonged to 14 days, somatic embryos at different stages of development (globular-, heart-, torpedo- and early and late cotyledonary shaped) were visible (Fig. 2c, d), and some of them spontaneously germinated on the same regeneration medium (e.g. 20 µM TDZ + 2 µM NAA, Fig. 2e). On BA-containing media, especially at 20 µM BA + 2 µM NAA and 30 µM BA + 3 µM NAA, fasciations of somatic embryos at the hypocotyl/root level occurred frequently (Fig. 2f), compared with other media containing TDZ or KIN.

The effect of PGRs on somatic embryogenesis and shoot regeneration

The control hypocotyl-slice explants cultured on MS medium without PGRs did not regenerate morphogenic structures (Fig. 3a, b). Although morhogenic structures regeneration started during first week of cultivation on
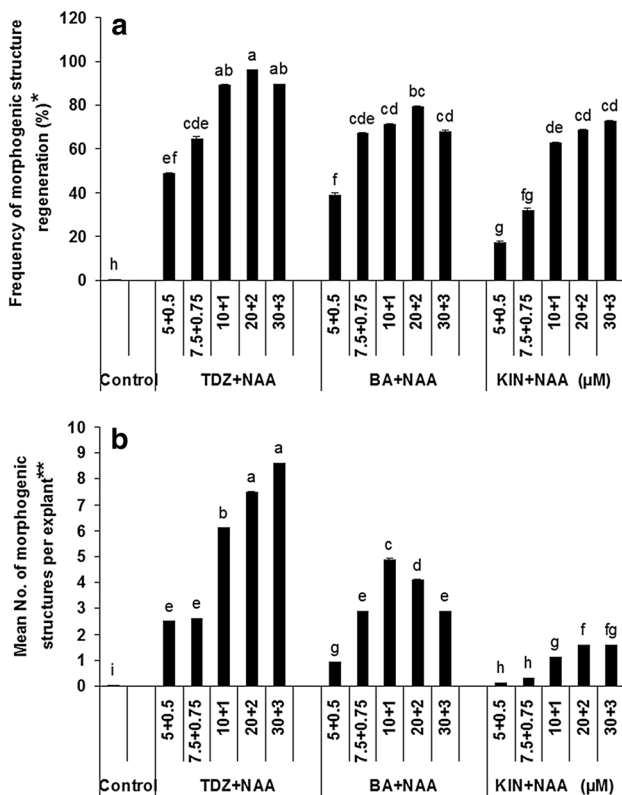

Fig. 3 Effect of type and concentration of cytokinins (CKs), in combination with NAA, on frequency of morphogenic structure regeneration from hypocotyl cross-section explants of collard greens (**a**), and on the mean number of morphogenic structures regenerted per explant (**b**) after 3 weeks of culture. *Number of morphogenic structure regenerating explants/total number of explants × 100. **Number of morphogenic structures per explant/total number of explants. Treatments denoted by different letters are significantly different ($P \leq 0.05$) according to the LSD test. Data represent mean values of three independent experiments ± SE

cytokinin/NAA-containing media, maximum of explant responsiveness was detected after 3 weeks of culture, when the majority of these structures were at the stage visible for counting with the aid of a stereomicroscope. Data of the number of somatic embryos and adventitious shoots were pooled together as these morphogenic structures in some cases have similar and undistinguishable appearance at this stage; therefore, separate counting was impossible. Results indicated that the genotype at the individual seedling level had the considerable effect on regeneration response, since the broad spectrum was observed among them (individual frequency ranging even from 0 to 100%, data not shown).

Therefore, to estimate the effect of cytokinins on morphogenic structure regeneration response, the high number of seedlings (455) had to be included and, accordingly, three independent experiments were performed. Since the regeneration response results of all of the three experiments conducted followed the similar trend, they were pooled for statistical purposes. The results of the regeneration response of 5267 hypocotyl slices, after 3 weeks of culture on the media containing different cytokinins (TDZ, BA or KIN) + NAA, are presented in Fig. 3. No regularity in the regeneration response rate related to the position of the cross section explant in hypocotyl was observed (data not shown). ANOVA indicated that the both cytokinin type and concentration, significantly affected morphogenic structure regeneration frequency, while their interaction was not significant. Data indicated the strongest effect of TDZ on morphogenic structure regeneration frequency, followed by the medium range effect of BA and by lowest effect of KIN (Fig. 3a). With each cytokinin used, regeneration frequency was increased along with the concentration increase. However, with higher cytokinin concentrations, there were mostly no significant differences between frequencies achieved on the MS media at 10, 20 and 30 µM (Fig. 3a). The highest morphogenic structure regeneration frequency was obtained using 20 µM TDZ, in combination with 2 µM NAA (95.9%), while 10/1 µM and 30/3 µM TDZ/NAA combination induced lower but statistically insignificant regeneration frequencies (Fig. 3a).

According to ANOVA, the mean number of morphogenic structures per explant was significantly affected by both cytokinin type and concentration, and by their interaction as well and followed the same trend as in the case of regeneration frequency TDZ > BA > KIN. Combinations with TDZ/NAA at 20/2 µM and 30/3 µM provided the highest number of morphogenic structures per explant (7.5 and 8.6, respectively, Fig. 3b).

Taking into the account the effectiveness of morphogenic structure regeneration, reported by the frequency of regeneration and the mean number of morphogenic structures per explant, and the appearance and viability of regenerated plants observed, the combination 20 µM TDZ + 2 µM NAA

could be selected as the optimal for regeneration of morphogenic structures from hypocotyl slices in collard greens.

Proliferation of somatic embryos

When distinct primary somatic embryos, regenerated on 20/2 µM of either TDZ, BA or KIN/NAA, were excised from the initial explant and transferred to the MS proliferation medium, containing 5 µM of the same cytokinin type (TDZ, BA or KIN, respectively), multiple secondary somatic embryos developed at the basis (Fig. 4a) or on the hypocotyl of primary embryo forming clusters within 3 weeks (Fig. 4b). On control, PGR-free MS medium, no somatic embryo proliferation was observed (Table 1). Also, the majority of primary somatic embryos cultivated on KIN-containing medium responded with weak (Table 1), or no secondary embryo proliferation, along with embryo yellowing and/or browning. Therefore, this medium was unsuitable for multiplication purpose. On the other hand, somatic

Table 1 Effect of 5 µM TDZ, BA or KIN on somatic embryo proliferation of *Brassica oleracea* L. var. *acephala* after 3 weeks of culture

Cytokinin (5 µM)	No. of explants	Mean No. of secondary embyos	Hyperhydration (%)	Fusion (%)
Control 0 µM	80	1.00 ± 0.000c	0	0
TDZ	85	7.79 ± 0.004a	9.3	2.7
BA	91	8.24 ± 0.004a	43.3	5
KIN	79	2.43 ± 0.002b	2.0	0

Data represent mean values of three replicates ± SE. Treatments denoted by the same letter in a column are not significantly different ($P \leq 0.05$) according to the LSD test

embryo explants displayed satisfactory proliferation rate on both TDZ- and BAcontaining media (7.79 and 8.24 shoots, respectively) (Table 1). However, proliferation of somatic embryos on BA-containing medium influenced negatively the appearance of regenerated plantlets by inducing leaf
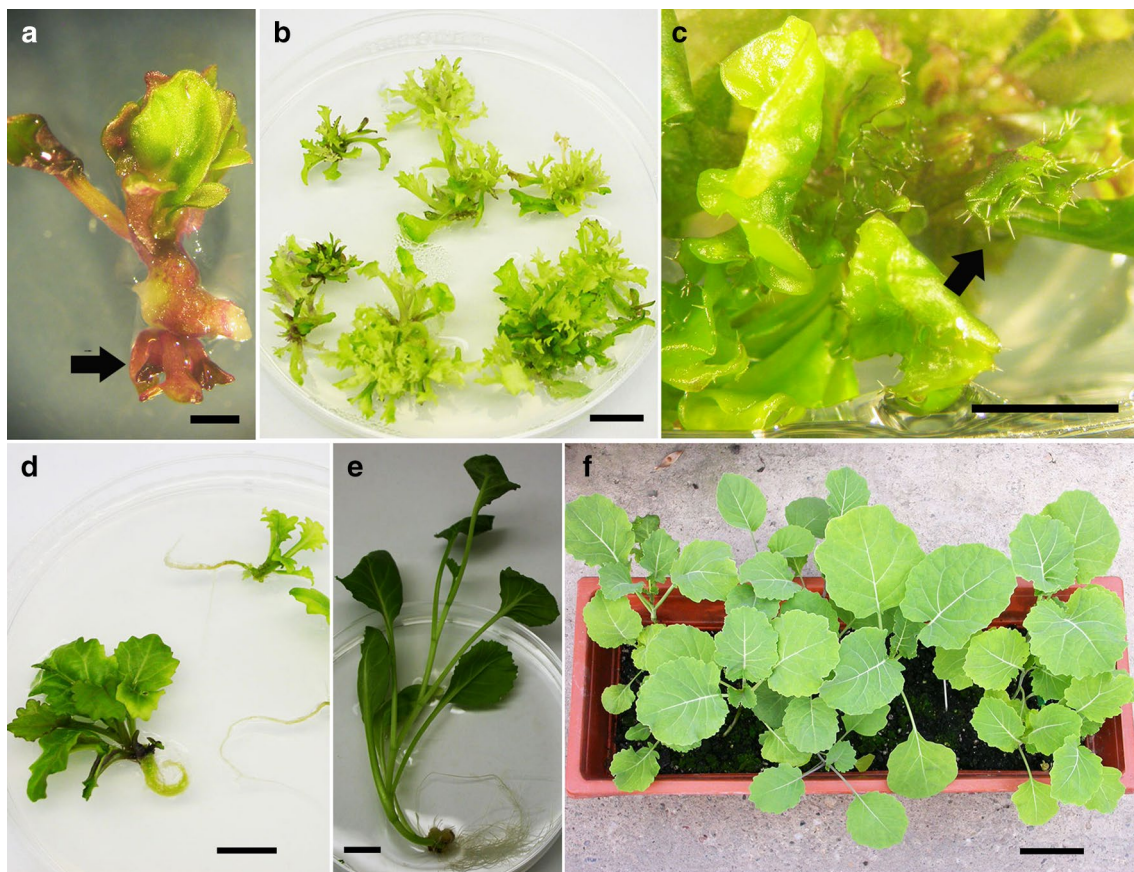

Fig. 4 Somatic embryos proliferation, growth and acclimatisation. **a** Multiple secondary somatic embryos developed at the basis (arrow) of primary embryo somatic embryo explant. **b** Multiple secondary embryo-derived plantlet clusters propagated for 3 weeks on MS medium containing 5 µM TDZ. **c** Trichome formation (arrow) on the leaf border of secondary somatic embryo-derived plantlets propa-gated for 3 weeks on MS medium containing 5 µM BA. **d** Development of somatic embryo-derived plantlet on PGR-free MS medium. **e** Individual rooted plant before transfer into the plastic container for acclimatisation. **f** Plants transferred to the plastic pot containing a 1:1 mix of soil and perlite and acclimatised for 6 weeks. Bars = 2 mm (**a**), 10 mm (**b**–**e**) and 50 mm (**f**)

trichome formation (Fig. 4c). Since secondary somatic embryos proliferated on TDZ-containing medium exhibited far-lower rates of hyperhydration and fusion than those cultivated on BA-containing medium (Table 1), TDZ is considered the most appropriate cytokinin for somatic embryo proliferation of collard greens.

Rooting and acclimatisation

After proliferation on MS media containing different cytokinins, the secondary somatic embryos (3.2 ± 0.5 cm in length) were separated and individually transferred to PGR-free MS solid medium, where they started to grow and developed roots (Fig. 4d). Seedlings germinated on PGR-free MS solid medium were used as a control, and, during an additional 3 weeks of culture on the same (PGR-free MS) medium in glass jars, these shoots grew vigorously and their roots elongated. The embryo-derived plantlets, which proliferated on the medium with KIN, displayed 100% rooting, as well as control seedlings, while those obtained from BA- and TDZ-containing media produced roots with lower frequencies (89.0 and 93.3% respectively) (Table 2). Besides rooting percentage, proliferation treatment had significant effect on the shoot length, while the effects on the number of roots per shoot and on the length of longest root were insignificant (Table 2). Plants (5–10 cm in length) with well-expanded leaves and roots (Fig. 4e) were transplanted into plastic containers and cultivated in the greenhouse. Regardless of proliferation medium used, most of the plants were successfully acclimatised to ex vitro conditions (Fig. 4f) with a high survival rate (99%). Based on visual inspection, the plants were healthy-looking and there was no morphological differences compared with the control seed-derived plants.

Total phenolic content, free radical scavenging activity, and HPLC profile of flavonoids in acclimatised in vitro-regenerated and seed-derived plants

The total phenolic content in leaves of acclimatised somatic embryo-derived collard greens plants varied from 25.96 to 44.90 mg GAE g^{-1} DW and according to ANOVA depended on cytokinin treatment used for regeneration/proliferation treatments (Table 3). Only TDZ treatment enhanced level of total phenolics in acclimatised plants (44.90 mg GAE g^{-1} DW) compared to seed-derived control plants (37.10 mg GAE g^{-1} DW). On the other hand, when other two cytokinins, BA and KIN, were applied for regeneration and proliferation of somatic embryos, a significant decrease of total phenolic content (27.24 and 25.96 mg GAE g^{-1} DW, respectively) compared to seed-derived control plants was observed (Table 3). Although HPLC analysis of the methanolic extract obtained from the in vitro-cultured plantlets, regenerated from different cytokinin treatments, revealed the presence of flavonoid compounds in very low amounts (data not shown), the higher amount of many different flavonoid glycosides was detected in leaves of the somatic embryo-derived acclimatised plants and seed-derived collard plants (Fig. 5, dashed line). Using

Table 2 Development of somatic embryos that have been proliferated on media with 5 μM TDZ, BA or KIN for 3 weeks and subsequently grown on PGR-free MS medium for an additional 6 weeks

Multiplication treatment (5 μM each)	No. of explants	Rooting percentage	Shoot length (cm)	Mean No. of roots	Length of longest root (cm)
TDZ	31	$93.3 \pm 2.1b$	$5.4 \pm 0.42c$	$4.1 \pm 0.052b$	$6.2 \pm 1.0a$
BA	28	$89.0 \pm 0.1b$	$8.6 \pm 0.50ab$	$6.4 \pm 0.054ab$	$7.0 \pm 0.8a$
KIN	29	$100 \pm 0.0a$	$7.2 \pm 0.54b$	$7.9 \pm 0.050a$	$5.9 \pm 0.5a$
MS (control)	27	$100 \pm 0.0a$	$8.7 \pm 0.63a$	$7.5 \pm 0.018ab$	$8.2 \pm 0.9a$

Data represent mean values of three replicates $\pm$ SE. Treatments denoted by the same letter in a column are not significantly different ($P \leq 0.05$) according to the LSD test

Table 3 The content of total phenolics (TPC), quercetin, and kaemferol and free radical scavenging activity (IC_{50}) of methanolic extracts of leaves of acclimatised collard greens plants obtained in vitro from somatic embryos from different cytokinin treatments and control seed-derived plants

Extract sample	Cytokinin treatment (5 μM)	TPC (mg GAE g^{-1} DW)	Quercetin (mg g^{-1} DW)	Kaempferol (mg g^{-1} DW)	IC_{50} (μg ml^{-1})
Control	0	$37.10 \pm 0.4b$	$0.165 \pm 0.01a$	$1.351 \pm 0.09a$	$24.58 \pm 0.4c$
Somatic embryo-derived plants	TDZ	$44.90 \pm 0.7a$	$0.078 \pm 0.03c$	$1.239 \pm 0.08ab$	$19.09 \pm 0.3d$
	BA	$27.24 \pm 0.3c$	$0.151 \pm 0.01ab$	$1.562 \pm 0.11a$	$27.20 \pm 0.5b$
	KIN	$25.96 \pm 0.5d$	$0.109 \pm 0.03bc$	$0.921 \pm 0.19c$	$48.94 \pm 0.4a$

Three samples were measured per treatment and the mean values were calculated and presented $\pm$ SE. Treatments denoted by the same letter in a column are not significantly different ($P \leq 0.05$) according to the LSD test
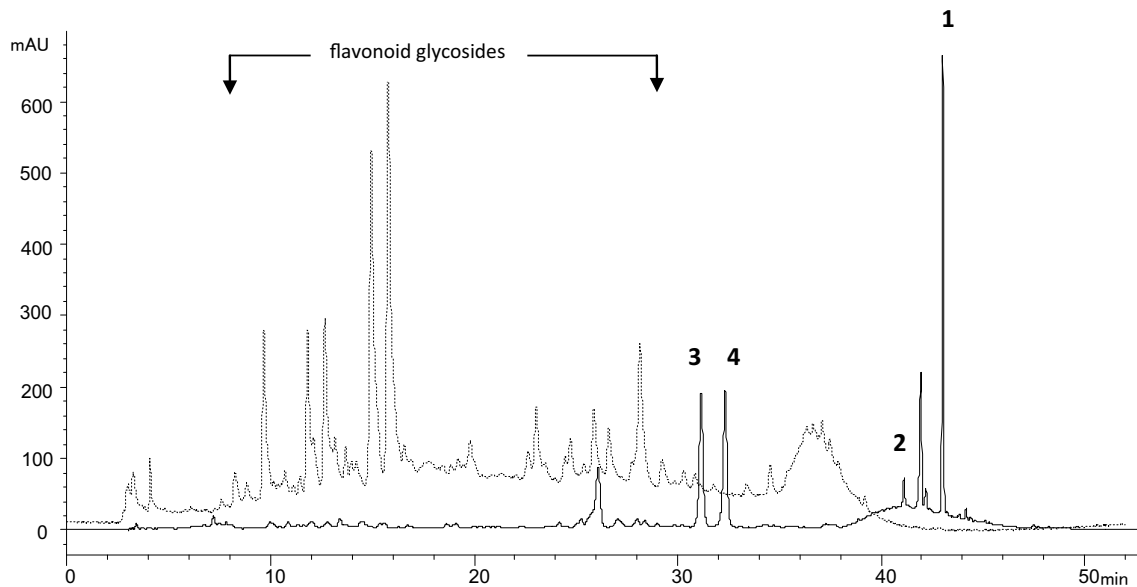
Fig. 5 Overlaid HPLC profiles ($\lambda = 360$ nm) of collard greens extracts of acclimatised plants obtained from TDZ treatment before acid-hydrolysis (dashed line) and after acid-hydrolysis (full line). Peak 1—kaempferol, peak 2—quercetin, peaks 3,4—unidentified flavonoid aglycons

acid-hydrolisys of the extract, the simplified HPLC profiles were obtained (Fig. 5, full line), and some of flavonoid aglycons, kaemferol and quercetin, were identified and quantified (Table 3).

The comparative HPLC analysis of methanolic extracts of these acclimatised plants showed no differences in qualitative composition between analyzed extracts of plants from different treatments, which were characterized by the presence of flavonoid aglycons quercetin (retention time $Rt = 41.1$ min) and kaempferol (dominant peak at $Rt = 43$ min). Two other detected but unidentified peaks ($Rt = 30$–34 min) were recognized as flavonoids, based on their UV spectra and the absorption maxima (Fig. 5). On the other hand quantitative analysis showed the highest quercetin content recorded in control and BA-derived plants, while there was not significant differences in kaempferol content between control and plants regenerated on BA and TDZ cytokinin treatment (Table 3). Methanolic extract of TDZ-treated plants, that contained the highest amount of total phenolics, also showed the highest radical scavenging activity value ($IC_{50} = 19.09$ µg ml^{-1}) (Table 3). The relation between total phenolic content and antiradical activity was observed with statistically significant correlation ($R^2 = 0.758$).

Discussion

The effect of PGRs on regeneration from hypocotyl slices

The present study describes a simple, one-step regeneration protocol for collard greens, established in hypocotyl slices from 5-day-old in vitro-grown seedlings. Seedling hypocotyls are preferred for regeneration and transformation of a number of *Brassica* species (Li et al. 2005; Sretenović-Rajičić et al. 2006; Munshi et al. 2007; Rafat et al. 2010; Akmal et al. 2011; Gerszberg et al. 2015). However, hypocotyls cut into several pieces provide the higher number of explants obtained from a single seedling. It could be especially favorable when a larger number of plants is required from the unique genotype. In addition, Ghnaya et al. (2008) reported that many thin slices from cotyledons and hypocotyls are suitable solution to speed up regeneration process in responsive species. PGRs were essential for the induction of regeneration in collard greens since no regeneration was achieved on PGR-free media. Among different cytokinin (BAP, TDZ or KIN)/

NAA combinations used, the combinations with TDZ had more effect on regeneration compared to BA and KIN. TDZ and BA are the most used cytokinins in Brassicas, but results of their effectiveness on regeneration are contradictory and depend on *Brassica* species, variety, or even genotype, as well as explant type used for induction of regeneration. As BA was optimal for range of *Brassica* species (Metz et al. 1995; Jin et al. 2000; Munshi et al. 2007; Sretenović-Rajičić et al. 2007; Maheshwari et al. 2011), TDZ was found to increase shoot regeneration in recalcitrant species (Cardoza and Stewart 2004; Jonoubi et al. 2005; Guo et al. 2005; Gambhir and Srivastava 2015) while KIN was used less (Kim et al. 2001). Using this one-step regeneration protocol for collard greens regeneration (20 μM TDZ + 2 μM NAA) it was possible to generate 402 regenerants from hypocotyl slices of one of the most responsive lines of collard greens after 3 weeks of culture, with 5.5% of hyperhydration only. For comparison, in the same time, one of the responsive lines produced 257 regenerants on the 20 μM BA + 2 μM NAA treatment, with much higher hyperhydration frequency of 22%. The benefit of implementation of the recommended protocol is simultaneous initiation of the both—shoot organogenesis and somatic embryogenesis. Somatic embryos represent prospective material for propagation and genetic transformation purposes as well since usually no chimaeric plants resulted from them. Somatic embryogenesis has never before been reported in collard greens. The occurrence of both regeneration pathways simultaneously, from identical in vitro conditions, has already been reported in some other species (Castillo et al. 2000; Madden et al. 2005; Bassuner et al. 2007). The effectiveness of TDZ on shoot regeneration within a narrow concentration range is known in many plant species, including Brassicas. Usually, it induced adventitious shoots at low concentrations (0.05–2.5 μM) and somatic embryos at a higher concentration (2.5–20 μM), depending on plant species (Singh et al. 2003; Gulshan et al. 2008; Mithila et al. 2003). Accordingly, Gaj (2004) reported that development pathways, somatic embryogenesis and organogenesis, could be separated by the use of the appropriate type and the concentration of PGR. With respect to these results, the effect of TDZ in collard greens was unexpected, since both organogenesis and somatic embryogenesis have been stimulated at wide range of concentrations, while the high effectiveness was achieved with the highest (20 and 30 μM) ones. In other plant species TDZ concentrations higher than 20 μM are usually inhibitory (Ning et al. 2007). As was observed, the position of the explant in the hypocotyl of collard greens did not influence regeneration, contrary to some other species such as *Linum usitatissimum* where explants excised from top part of the seedling, just below

cotyledons, displayed the highest shoot regeneration ability (Yildiz 2012).

Histology of somatic embryogenesis and shoot organogenesis from hypocotyl slices

The embryogenic and organogenic morphogenetic pathway in collard greens was confirmed by histological analysis, indicating that adventitious shoot formation was uniformly distributed within central cylinder, or were restricted to its cambial region, similarly as shown in the transverse section of hypocotyls of *Albizia odoratissima* (Rajeswari and Paliwal 2008). In collard greens, somatic embryos formed mainly from the cortical region of the hypocotyl-slice explant. Adventitious shoot organogenesis and somatic embryogenesis occurring simultaneously from identical in vitro conditions have already been reported in some species (Castillo et al. 2000; Madden et al. 2005; Bassuner et al. 2007). Regeneration via both regeneration pathways was obtained by TDZ treatment of leaf and petiole explants in *Saintpaulia ionantha* Wendl. (Mithila et al. 2003) and leaf tissues of *Rosa* L. hybrid cultivars (Li et al. 2002); while using a TDZ + IAA combination a number of different structures were regenerated from leaf explants of *Elliottia racemosa* (Woo and Wetzstein 2008). Such observation indicates that the regeneration pathway might vary with explant tissue, which could be dependent on the endogenous hormone content and on the cytokinins/auxin ratio in a tissue cells. Features of the most of the regenerated structures, such as the presence of both shoot and root pole and the absence of vascular connection with callus tissue, explicitly indicated their embryogenic nature. The fusion of embryos at their root/hypocotyl levels was the main abnormality, mainly observed on BA-containing media, and the emergence of embryos from callus by the root pole, as well as their precocious germination, is not unexpected, since they have been observed in other varieties of the *B. oleracea* species, e.g. cabbage, cauliflower or kohlrabi (Pavlović et al. 2013; Ćosić et al. 2013).

Somatic embryos proliferation and secondary metabolites production in regenerants

Although TDZ and BA influenced equally collard greens somatic embryos proliferation, TDZ-treated somatic embryos displayed less malformation effects, like as hyperhydration and fusion, than BA-treated, without adverse effects on subsequent rooting. Observed trichome inducing effect of medium with higher BA concentration is not suprising since that effect of BA has already been recorded and studied extensivelly in the Brassica model *Arabidopsis thaliana* (Maes and Goossens 2010). Hence, TDZ is also a cytokinin of choice, concerning somatic embryos

multiplication. Apart from their beneficial role in regeneration process, cytokinins may affect production of secondary metabolites in regenerated plants. Such effect can be positive, as in *Scutellaria alpina*, where 0.5 µM TDZ was the most effective for polyphenol production (Grzegorczyk-Karolak et al. 2015), or negative, as on hypericin production in *Hypericum* shoots (Liu et al. 2007; Coste et al. 2011). In somatic embryo-derived collard green plants, TDZ increased total phenolic content. However, the proof of the role of TDZ in increasing production of phenolic compounds is still fragmentary and requires further investigation. The amount of total phenolic compounds was higher in the leaves of the acclimatised plants, compared to in vitro-cultured plantlets. This result is in accordance with that of Bairu et al. (2011), who also found significantly higher total phenolic content in acclimatised compared to those in tissue-cultured *Harpagophytum procumbens* shoots, and in contrast to findings of Aremu et al. (2013) in *Merwilla plumbea*, and Jeong and Sivanesan (2015) in *Scrophularia takesimensis*. Phenolic compounds are known to possess multiple biological activities, but for edible plants their antioxidant and anti-inflammatory activity is the most important (Polya 2003; Wojdyło et al. 2007). Among them flavonoids, such as kaempferol and quercetin, identified in acclimatised collard plants, are the most widespread and diverse group of polyphenols in *Brassica* species. An important effect of flavonoids is the scavenging of oxygen-derived free radicals (Nijveldt et al. 2001). Since TDZ-derived plants did not contain increased kaempferol and quercetin amounts compared with plants regenerated from other treatments, their enhanced antioxidant activity could be attributed to other unidentified flavonoids (see Fig. 5, peaks 3 and 4).

Conclusion

In the present study, an efficient protocol for plant regeneration through somatic embryogenesis and shoot organogenesis from hypocotyl slices of collard greens has been developed. This is the first report on induction of somatic embryogenesis in collard greens. The type and the concentration of used cytokinins significantly affected regeneration. Using the optimal PGR combination tested − 20 µM TDZ + 2 µM NAA, the high regeneration response was achieved throughout both regeneration pathways, somatic embryogenesis and shoot organogenesis. TDZ at 5 µM provided efficient multiplication through secondary somatic embryos production and lower hyperhydration rate. The application of TDZ provoked total phenolic content increase along with improved antioxidant properties. The regeneration system described here via somatic embryogenesis could be useful for propagation of desirable collards genotypes. It can also provide available material for genetic

transformation, in order to improve collard greens plants, e.g to elevate nutritive value of this vegetable plant by modulation of the production of naturally occurring pigments or by enhanced production of polyphenols. On the other hand, the induction of embryogenesis and organogenesis in the same explant provide an appropriate experimental system to undertake comprehensive analysis of the physiological and developmental events which procure these morphogenic processes. Apart this, generated somatic embryos are prospective material for artificial seed production in this species.

Author contributions NB supervised the whole study and wrote the manuscript. BV performed hystological analysis. DKM performed phytochemical analysis. JM and LJT produced and maintained *in vitro* cultures. NG cultivated plants in greenhouse. SZK helped with experimental design and contributed to the writing and correction of the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

Akmal M, Nafis T, Mirza KJ, Alam P, Mohammad A, Mujib A, Abdin MZ (2011) High frequency somatic embryogenesis in mustard crop (*Brassica juncea* L. cv. Pusa Jai kisan): microscopic and histological analyses. Aust J Crop Sci 5:1783–1789

Al Shamari M (2014) Somatic embryogenesis and cryopreservation of cauliflower (*Brassica oleracea* var. *botrytis*). PhD thesis, School of Biological Sciences, Faculty of Science and Environment, University of Plymouth, UK

Aremu AO, Gruz J, Šubrtová M, Szüčová L, Doležal K, Bairu MW, Finnie JF, Van Staden J (2013) Antioxidant and phenolic acid profiles of tissue cultured and acclimatized *Merwilla plumbea* plantlets in relation to the applied cytokinins. J Plant Physiol 170:1303–1308

Bairu MW, Amoo SO, Van Staden J (2011) Comparative phytochemical analysis of wild and *in vitro*-derived greenhouse-grown tubers, *in vitro* shoots and callus-like basal tissues of *Harpagophytum procumbens*. S Afr J Bot 77:479–484

Bassuner BM, Lam R, Lukowitz W, Yeung EC (2007) Auxin and root initiation in somatic embryos of *Arabidopsis*. Plant Cell Rep 26:1–11

Brand-Williams W, Cuvelier ME, Berset C (1995) Use a free radical method to evaluate antioxidative activity. LWT - Food Sci Technol 28:25–30

Cao J, Earle ED (2003) Transgene expression in broccoli (*Brassica oleracea* var. *italica*) clones propagated in vitro via leaf explants. Plant Cell Rep 21:789–796

Cao J, Shelton AM, Earle ED (2005) Development of transgenic collards (*Brassica oleracea* L. var. *acephala*) expressing a *cry1Ac* or *cry1C* Bt gene for control of the diamondback moth. Crop Protect 24:804–813

Cardoza V, Stewart CN Jr (2004) Brassica biotechnology: progress in cellular and molecular biotechnology. In Vitro Cell Dev Biol Plant 40:542–551

Castillo P, Marquez J, Rubluo A, Hernandez G, Lara M (2000) Plant regeneration from callus and suspension cultures of *Valeriana edulis* ssp. *procera* via simultaneous organogenesis and somatic embryogenesis. Plant Sci 151:115–119

Ćosić T, Vinterhalter B, Vinterhalter D, Mitić N, Cingel A, Savić J, Bohanec B, Ninković S (2013) *In vitro* plant regeneration from immature zygotic embryos and repetitive somatic embryogenesis in kohlrabi (*Brassica oleracea* var. *gongylodes*). In Vitro Cell Dev Biol—Plant 49:294–303

Coste A, Vlase L, Halmagyi A, Deliu C, Coldea G (2011) Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tiss Organ Cult 106:279–288

Dai XG, Shi XP, Ye YM, Fu Q, Bao MZ (2009) High frequency plant regeneration from cotyledon and hypocotyl explants of ornamental kale. Biol Plant 53:769–773

Deo PC, Tyagi AP, Taylor M, Harding R, Becker D (2010) Factors affecting somatic embryogenesis and transformation in modern plant breeding. SPJNAS 28:27–40

Feng H, Guo S, Jiang FY (2009) Microspore-derived embryos and plant regeneration in edible kale (*Brassica oleracea* L. var. *acephala* DC.) Acta Hortic Sin 36:587–592

Ferreres F, Sousa C, Vrchovská V, Valentão P, Pereira JA, Seabra RM, Andrade PB (2006) Chemical composition and antioxidant activity of tronchuda cabbage internal leaves. Eur Food Res Technol 222:88–98

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.). Heynh. Plant Growth Regul 43:27–47

Gambhir G, Srivastava DK (2015) Thidiazuron induces high frequency shoot regeneration in leaf and petiole explants of cabbage (*Brassica Oleracea L. Var. Capitata*). J Biotechnol Biomater 5:172. https://doi.org/10.4172/2155-952X.1000172

Gerszberg A (2018) Tissue culture and genetic transformation of cabbage (*Brassicaoleracea* var. *capitata*): an overview. Planta 248:1037–1048

Gerszberg A, Hnatuszko-Konka K, Kowalczyk T (2015) *In vitro* regeneration of eight cultivars of *Brassica oleracea*. var. *capitata*. In Vitro Cell Dev Biol Plant 51:80–87

Ghasemzadeh A, Jaafar HZE, Rahmat A, Wahab PEM, Halim MRA (2010) Effect of different light intensities on total phenolics and flavonoids synthesis and anti-oxidant activities in young ginger varieties (*Zingiber officinale* Roscoe). Int J Mol Sci 11:3885–3897

Ghnaya AB, Charles G, Branchard M (2008) Rapid shoot regeneration from thin cell layer explants excised from petioles and hypocotyls in four cultivars of *Brassica napus* L. Plant Cell Tiss Organ Cult 92:25–30

Gong Y, Sohn H, Xue L, Firestone GL, Bjeldanes LF (2006) 3,3′-Diindolylmethane is a novel mitochondrial H+-ATP synthase inhibitor that can induce p21$^{Cip1/Waf1}$ expression by induction of oxidative stress in human breast cancer cells. Cancer Res 66:4880–4887

Grzegorczyk-Karolak I, Kuźma Ł, Wysokińska H (2015) The effect of cytokinins on shoot proliferation, secondary metabolite production and antioxidant potential in shoot cultures of *Scutellaria alpina*. Plant Cell Tiss Organ Cult 122:699–708

Gulshan C, Darshna C, Madan V, Manish S, Pawan KJ (2008) TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). Physiol Mol Biol Plants 14:347–353

Guo DP, Zhu ZJ, Hu XX, Zheng SJ (2005) Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of team mustard (*Brassica juncea* var. *tsatsai*). Plant Cell Tiss Organ Cult 83:123–127

Jeong B, Sivanesan I (2015) Direct adventitious shoot regeneration, in vitro flowering, fruiting, secondary metabolite content and antioxidant activity of *Scrophularia takesimensis* Nakai. Plant Cell Tiss Organ Cult 123:607–618

Jin RG, Liu YB, Tabashnik B, Borthakur D (2000) Development of transgenic cabbage (*Brassica oleracea* var. *capitata*) for insect resistance by *Agrobacterium tumefaciens*-mediated transformation. In Vitro Cell Dev Biol Plant 36:231–237

Jonoubi P, Mousavi A, Majd A, Salmanian AH, Javaran MJ, Daneshian J (2005) Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*. Biol Plant 49:175–180

Kahlon TS, Chiu M-CM, Chapman MH (2008) Steam cooking significantly improves in vitro bile acid binding of collard greens, kale, mustard greens, broccoli, green bell pepper, and cabbage. Nutr Res 28:351–357

Kim NR, An G, Park MC (2001) High-frequency regeneration and transformation of *Raphanus sativus*. J Plant Biol 44:231–235

Lännenpää M (2014) Heterologous expression of AtMYB12 in kale (*Brassica oleracea* var. *acephala*) leads to high flavonol accumulation. Plant Cell Rep 33:1377–1388

Li X, Krasnyanski SF, Korban SS (2002) Somatic embryogenesis, secondary somatic embryogenesis, and shoot organogenesis in *Rosa*. J Plant Physiol 159:313–319

Li X, Peng RH, Fan HQ, Xiong AS, Yao QH, Cheng ZM, Li Y (2005) *Vitreoscilla* hemoglobin overexpression increases submergence tolerance in cabbage. Plant Cell Rep 23:710–715

Lillo C, Olsen JE (1989) Growth and shoot formation in protoplast-derived calli of *Brassica oleracea* ssp. *acephala* and ssp. *capitata*. Plant Cell Tiss Organ Cult 17:91–100

Liu XN, Zhang XQ, Sun JS (2007) Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. Plant Growth Regul 53:207–214

Macalalad EA, Robidillo CJT, Marfori EC (2016) Influence of different cytokinins on the growth, [6]-Gingerol production and antioxidant activity of *in vitro* multiple shoot culture of ginger (*Zingiber officinale* Roscoe). Res J Med Plant 10:194–200

Madden JI, Jones CS, Auer CA (2005) Modes of regeneration in *Pelargonium x hortorum* (Geraniaceae) and three closely related species. In Vitro Cell Dev Biol- Plant 41:37–46

Maes L, Goossens A (2010) Hormone-mediated promotion of trichome initiation in plants is conserved but utilizes species- and trichome-specific regulatory mechanisms. Plant Signal Behav 5:205–207

Maheshwari P, Selvaraj G, Kovalchuk I (2011) Optimization of *Brassica napus* (canola) explant regeneration for genetic transformation. Nat Biotechnol 29:144–155

Manchali S, Murthy KNC, PatilBS (2012) Crucial facts about health benefits of popular cruciferous vegetables. J Funct Foods 4:94–104

Metz TD, Dixit R, Earle ED (1995) *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var. *italica*) and cabbage (*B. oleracea* var. *capitata*). Plant Cell Rep 15:287–292

Miller-Cebert RL, Sistani NA, Cebert E (2009) Comparative mineral composition among canola cultivars and other cruciferous leafy greens. J Food Compos Anal 22:112–116

Mithila J, Hall JC, Victor JMR, Saxena PK (2003) Thidiazuron induces shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet (*Saintpaulia ionantha* Wendl.). Plant Cell Rep 21:408–414

Munshi MK, Roy PK, Kabir MH, Ahmed G (2007) In vitro regeneration of cabbage (*Brassica oleracea* L. var. *capitata*) through hypocotyl and cotyledon culture. Plant Tissue Cult Biotechnol 2:131–136

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418–425

Ning GG, Bai SP, Bao MZ, Liu L (2007) Factors affecting plantlet regeneration from *in vitro* cultured immature embryos and cotyledons of *Prunus mume* "Xue mei". In Vitro Cell Dev Biol Plant 43:95–100

Niu RQ, Zhang Y, Tong Y, Liu ZY, Wang YH, Feng H (2015) Effects of *p*-chlorophenoxyisobutyric acid, arabinogalactan, and activated charcoal on microspore embryogenesis in kale. Genet Mol Res 14:3897–3909

NYSVegExpo (2017) http://www.hort.cornell.edu/expo/proceedings/2017/Colecrops_Breeding%20Swegarden_N. YSVegExpo_Jan2017.pdf

Olson SM, Freeman JH (2008) Collard cultivar evaluations in northern Florida. HortTechnology 18:536–538

Pavlović S, Vinterhalter B, Zdravković-Korać S, Vinterhalter D, Zdravković J, Cvikić D, Mitić N (2013) Recurrent somatic embryogenesis and plant regeneration from immature zygotic embryos of cabbage (*Brassica oleracea* var. *capitata*) and cauliflower (*Brassica oleracea* var. *botrytis*). Plant Cell Tiss Organ Cult 113:397–406

Pereira JV, Santos HB, Agra MF, Guedes DN, Modesto-Filho J (2006) Use of cabbage leaves *(Brassica oleracea* var. *acephala)* in the stabilization of bone mass after menopause. Braz J Pharmacognosy 16:345–349

Pogrebnyak N, Markley K, Smirnov Y, Brodzik R, Bandurska K, Koprowski H, Golovkin M (2006) Collard and cauliflower as a base for production of recombinant antigens. Plant Sci 171:677–685

Polya GM (2003) Biochemical targets of plant bioactive compounds. A pharmacological reference guide to sites of action and biological effects. CRC Press, Boca Raton

Puddephat J, Riggs TJ, Fenning TM (1996) Transformation of *Brassica oleracea* L.: a critical review. Mol Breed 2:185–210

Qamar Z, Hossain MB, Nasir IA, Tabassum B, Husnain T (2014) *In vitro* development of cauliflower synthetic seeds and development of plantlets in vivo. Plant Tiss Cult Biotech 24:27–36

Rafat A, Aziz MA, Rashid AA, Abdullach SNA, Kamaladini H, Sirchi MHT, Javadi MB (2010) Optimization of *Agrobacterium tumefaciens*-mediated transformation and shoot regeneration after co-cultivation of cabbage (*Brassica oleracea* subsp. *capitata*) cv. KY Cross with AtHSP101 gene. Sci Hortic 124:1–8

Rajeswari V, Paliwal K (2008) In vitro adventitious shoot organogenesis and plant regeneration from seedling explants of *Albizia odoratissima* L.f. (Benth.). In Vitro Cell Dev Biol Plant 44:78–83

Singh ND, Sahoo L, Sarin NB, Jaiwal PK (2003) The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Millsp). Plant Sci 164:341–347

Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. Am J Enol Vitic 16:144–158

Sretenović-Rajičić T, Ninković S, Miljuš-Dukić J, Vinterhalter B, Vinterhalter D (2006) *Agrobacterium rhizogenes*-mediated transformation of *Brassica oleracea* var. *sabauda* and *B. oleracea* var. *capitata*. Biol Plant 50:525–530

Sretenović-Rajičić T, Ninković S, Uzelac B, Vinterhalter B, Vinterhalter D (2007) Effects of plant genotype and bacterial strain on *Agrobacterium tumefaciens*-mediated transformation of *Brassica oleracea* L. var. *capitata*. Russ J Plant Physiol 54:653–658

Taghizadeh M, Solgi M (2015) Introduction of commercial protocol for in vitro proliferation of *Brassica oleracea* var. *acephala*. Iranian J Hortic Sci 45:475–484

USDA (2012) from https://www.nass.usda.gov/Publications/AgCensus/2012/Full_Report/Volume_1,_Chapter_1_US/usv1.pdf

Wang Y, Tong Y, Li Y, Zhang Y, Zhang J, Feng J, Feng H (2011) High frequency plant regeneration from microspore-derived embryos of ornamental kale (*Brassica oleracea* L. var. *acephala*). Sci Hortic 130:296–302

Wojdyło A, Oszmiański J, Czemerys R (2007) Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem 105:940–949

Woo SM, Wetzstein HY (2008) An efficient tissue culture regeneration system for Georgia plume, *Elliottia racemosa*, a threatened Georgia endemic. HortSci 43:447–453

Xiushu Z, Mingyang L, Wenling Z, Fan L (2009) Establishment of high adventitious shoot regeneration system of ornamental kale. Genomics Appl Biol 28:141–148

Yildiz M (2012) The prerequisite of the success in plant tissue culture: high frequency shoot regeneration. In: Leva A, Rinaldi LMR (eds) Recent advances in plant in vitro culture. Intech, Rijeka, pp 63–90