

Improvements of doubled haploid production protocol for white cabbage (*Brassica oleracea* var. *capitata* L.)

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ABSTRACT

Protocols leading to the development of doubled haploid (DH) lines by microspore culture are widely used in white cabbage (*Brassica oleracea* var. *capitata* L.), but efficiency varies according to the cultivar and induction procedure. Forty different genotypes consisting of F1 cultivars and their crosses with responsive doubled haploid lines were tested to evaluate the androgenic response. In total, 20,032 embryos were produced. On average, the haploid induction response of F1 cultivars was 7.0 embryos/Petri dish, but the average of these hybrids crossed to responsive DH lines was 26.6 embryos/Petri dish. In seven reciprocal crosses, a difference was observed in just one, meaning that the maternal effect probably has a minor influence on haploid embryogenesis in cabbage. Addition of 0.02% activated charcoal (AC) to the induction media increased embryo formation in several low-responsive genotypes, but its effect on embryo formation of high-responsive genotypes was predominantly negative, although larger embryos were formed on media containing AC than without AC. Further development into plantlets was tested by two procedures. Formed embryos were either transferred directly to regeneration medium or treated with abscisic acid and desiccated for 4 weeks. Regrowth and further development reached on average 15.5 and 57.6%, for the first and second procedures, respectively. Plantlets developed by direct transfer often exhibited abnormal development or hyperhydricity, unlike the desiccated embryos. Spontaneous diploidisation of embryos reached 42.5% in total and was not affected by AC added to the induction media.

Key words: activated charcoal, effect of genotypes, microspore culture, ploidy level

INTRODUCTION

White cabbage (*Brassica oleracea* var. *capitata* L.) is the most important vegetable within the *B. oleracea* group, with a worldwide annual production of 71.7 million tons (FAOSTAT, 2014). Due to their uniformity and higher yields, F1 hybrid varieties dominate on the seed market. Hybrid cultivars require the production of inbred lines, which can be achieved by self-pollination or by doubled haploid (DH) induction. This last protocol has several advantages, such as short time needed to

produce inbred lines and complete homozygosity. In brassicas, anther and microspore cultures both lead to haploid induction. However, during the last two decades, microspore culture has predominated as an optimal procedure in white cabbage. Rudolf et al. (1999) established an advanced protocol for the induction of haploid embryos and their germination, but focused on the genotype-related response. Since then, several attempts have been made to improve the existing method. Yuan et al. (2012) demonstrated that increased pH in combination with

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the addition of 2-(*N*-morpholino) ethanesulphonic acid and arabinogalactan-protein increased embryo production. Similarly, Zeng et al. (2015) tested the incorporation of ascorbic acid into the induction media and performed cell purification after 3 days in culture to increase the viability of the microspores. Both studies reported an increased embryo yield of previously recalcitrant cultivars.

Several additional factors have been investigated in other *B. oleracea* morphotypes. Notably, the influence of adding activated charcoal (AC) to the media tended to provide beneficial effects for nine morphotypes of *B. oleracea* (Dias, 1999), cauliflower (Bhatia et al., 2016) and further members of the Brassicaceae family, such as *Brassica juncea* (Prem et al., 2008) and *Eruca sativa* (Leskovšek et al., 2008), where it was found essential for obtaining reproducible results.

When microspore-derived embryos are formed, a majority undergo abnormal development when placed directly on regeneration media without some pre-treatment. In such instances, abnormal proliferation occurs, for example, hyperhydric swelling of the hypocotyl and cotyledons or, instead of shoot proliferation, secondary embryos are formed. As reviewed by Takahashi et al. (2012), direct plant regeneration from microspore-derived embryos is often low and variable in brassicas, though species differ regarding this characteristic. Microspore embryogenesis in the cited article varied among 22 responsive genotypes from 0.02 to 15.0 embryos for 2×10^5 microspores, while 2 genotypes produced no embryos. Gu et al. (2014) reported on a germination rate of around 30% by employing a hormone treatment (zeatin, indole-3-acetic acid, and 6-benzylaminopurine) without desiccation. It has been documented that application of abscisic acid (ABA), with or without desiccation, enhances the direct plant regeneration in several *Brassica* spp. (Kott and Beversdorf, 1990; Huang et al., 1991; Wakui et al., 1994; Rudolf et al., 1999; Zhang et al., 2006).

This study assessed a broad range of cultivars and their crosses with responsive DH lines (some in a reciprocal approach) on the responsiveness to haploid embryo induction. Particular focus was given to the effect of AC incorporated into the induction media on the generation of microspore-derived embryos and their subsequent development into plantlets. Spontaneous chromosome doubling efficiency, concerning the induction procedure, was scored.

MATERIAL AND METHODS

Plant materials and growth conditions

The plant materials used in the experiments are listed in Table 1. Selection of genotypes was made according to their agronomic performance and/or haploid induction potential scored in the previous years. Plants were grown in the field, excavated in autumn and vernalized at 4°C for 12 weeks in the dark, then placed in a greenhouse, where flowering was induced (March-May).

Microspore isolation and culture

For each experiment, 30 flower buds containing late uninucleate microspores were selected, as previously described (Rudolf et al., 1999), and surface-sterilised in 1.66% g l⁻¹ dichloroisocyanuric acid sodium salt for 6 min. and then washed three times in sterile distilled water. The microspores were released by gently crushing the buds in 2 ml of filter-sterilised hormone-free NLN-13 medium (Lichter, 1982) containing 13% sucrose. Next, the slurry was filtered through a double layer of 40-µm nylon mesh, with 38 ml NLN-13 medium added. The filtrate was centrifuged at 190 g for 3 min. After discarding the supernatant, the pellet of microspores was resuspended in 10 ml of NLN-13 medium. This procedure was repeated twice, for a total of three centrifugations and resuspensions. The obtained microspores (supernatant) were pooled and diluted with the same medium to the final concentration corresponding to one bud per ml. The adjusted microspore suspension was distributed into 50-ml sterile test tubes (half of them containing 0.02% AC, according to the experimental scheme) and incubated in the dark at 30°C for 48 h. Following this stress treatment, the microspore suspensions were centrifuged at 190 g for 4 min., the supernatant removed and replaced with fresh NLN-13 medium, before distribution in 50-mm Petri dishes. The dishes were incubated in a growth chamber at 25°C for 10 days in the dark and then transferred to an orbital shaker, operating at 40 rpm. The number of formed embryos was scored 33 days post-isolation and their size noted. For each microspore isolation (genotype and treatment), at least three repetitions with three Petri dishes were made.

Desiccation and plantlet growth

Formed embryos at cotyledonary stage (Fig. 1A) were either placed on regeneration medium without desiccation or were treated with abscisic acid (ABA-Duchefa) and desiccated. For ABA treatment, the procedure was as follows: ABA was

Table 1. List of genotypes used in microspore embryogenesis experiments

Genotype	Type of accession	Source
Atria F ₁	commercial hybrid	Seminis
Burton F ₁	commercial hybrid	Nickerson Zwaan
Krautman F ₁	commercial hybrid	Bejo Zaden
Presnik F ₁	commercial hybrid	Biotechnical Faculty, Ljubljana
Tacoma F ₁	commercial hybrid	Rijk Zwaan
Checkmate F ₁	commercial hybrid	Bejo Zaden
Benelli F ₁	commercial hybrid	Bejo Zaden
Tamarindo F ₁	commercial hybrid	Bejo Zaden
Fieldwinner F ₁	commercial hybrid	Bejo Zaden
Autumn Queen F ₁	commercial hybrid	Takii Seed
Agressor F ₁	commercial hybrid	Syngenta
Grandslam F ₁	commercial hybrid	Sakata
Atria F ₁ × 2	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × 4	experimental cross	Biotechnical Faculty, Ljubljana
4 × Atria F ₁	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × 5	experimental cross	Biotechnical Faculty, Ljubljana
5 × Atria F ₁	experimental cross	Biotechnical Faculty, Ljubljana
6 × Atria F ₁	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × 7	experimental cross	Biotechnical Faculty, Ljubljana
7 × Atria F ₁	experimental cross	Biotechnical Faculty, Ljubljana
8 × Atria F ₁	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × 92	experimental cross	Biotechnical Faculty, Ljubljana
92 × Atria F ₁	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × 165	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × (36 × 165)	experimental cross	Biotechnical Faculty, Ljubljana
Atria F ₁ × K.O.	experimental cross	Biotechnical Faculty, Ljubljana
Kranjsko okroglo (K.O.)	local Slovene open pollinated variety	Slovene gene bank
K.O. × 2	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × 4	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × 5	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × 6	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × 7	experimental cross	Biotechnical Faculty, Ljubljana
8 × K.O.	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × 92	experimental cross	Biotechnical Faculty, Ljubljana
92 × K.O.	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × 165	experimental cross	Biotechnical Faculty, Ljubljana
165 × K.O.	experimental cross	Biotechnical Faculty, Ljubljana
K.O. × (36 × 165)	experimental cross	Biotechnical Faculty, Ljubljana
278 × Burton F ₁	experimental cross	Biotechnical Faculty, Ljubljana

2, 4, 5, 6, 7, 8, 36, 92, 165, 278 are doubled haploid lines, produced in previous breeding programmes at Biotechnical Faculty in Ljubljana via microspore culture

dissolved in 70% ethanol, delivered to Petri dishes and evaporated, then NLN-13 medium was added to give a final ABA concentration of 5 mg l⁻¹. All obtained embryos were soaked in this solution for 1 day at 25°C in the dark on an orbital shaker (40 rpm) and then transferred into dry Petri dishes, containing moistened filter paper, for 4 weeks in the dark at 25°C. Fresh or desiccated embryos were then placed in growth chambers on B5 medium (Gamborg et al., 1968) with 20 g l⁻¹ sucrose at pH

5.8 under 16-h photoperiod at 23°C. Plantlets were scored for the appearance of hyperhydricity.

Determination of ploidy level

Ploidy level was determined on plantlets grown on regeneration medium for 40-50 days by flow cytometry using 4',6'-diamidino-2-phenylindole staining, according to Bohanec (2003). Measurements were done with a CyFlow space flow cytometer (Partec GmbH, Görlitz,

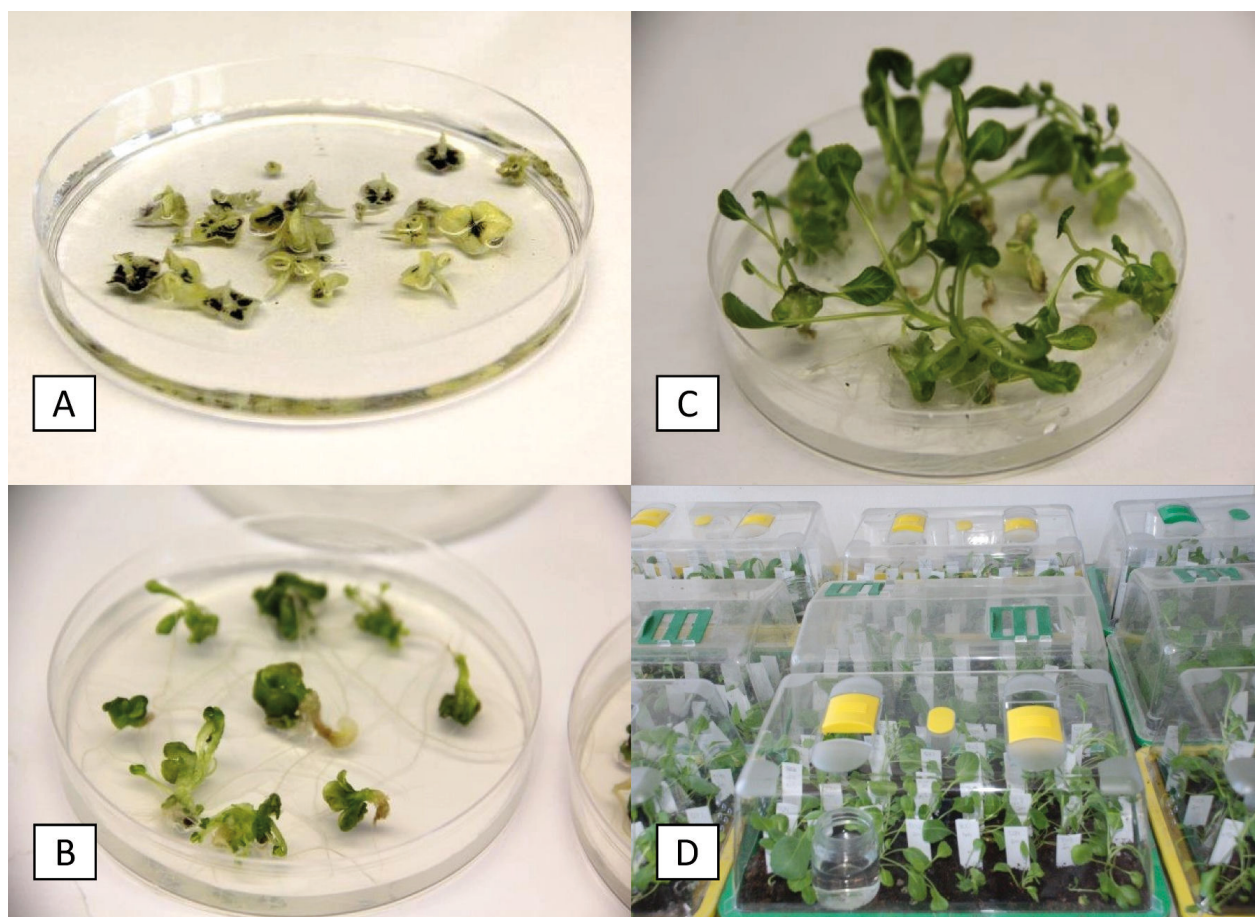


Figure 1. Developmental stages of microspore-derived embryos of white cabbage: (A) embryos formed in liquid media with the addition of activated charcoal; (B) deformed plantlets from embryos placed in a regeneration medium without desiccation; (C) normal plantlets originated from desiccated embryos; (D) acclimatisation of plantlets

Germany), using a linear scale with a diploid white cabbage genotype positioned at channel 200 as the external standard.

Statistical analysis

Analysis of variance (ANOVA) was calculated using Statgraphics Centurion XV.II statistical package.

RESULTS

Haploid induction ability of 40 varieties and experimental hybrids (cultivars crossed with doubled haploid lines with embryogenic potential)

was evaluated. Microspores were cultured on NLN-13 medium, with or without AC addition (Fig. 2). In total, 9,867 and 10,165 embryos were formed on media with and without AC, respectively. The results show a major influence of the genotypes on embryo formation ability, and crossing with DH lines increased the response more than threefold. However, as presented in Table 2, the effects of genotype and interactions were statistically significant at the 95% confidence level.

Overall, the variability in embryogenic response within genotype was high, especially in low-

Table 2. Analysis of variance for number of embryos/Petri dish

Source	Sum of squares	df	Mean square	F-ratio	p-value
Main effects					
A:treatment	1654	1	1654	3.7	0.0595
B:genotype	296104	39	7592	16.3	0.0000
Interactions					
AB	34108	39	875	1.8	0.0012
Residual	335058	719	466		
Total (corrected)	679212	798			

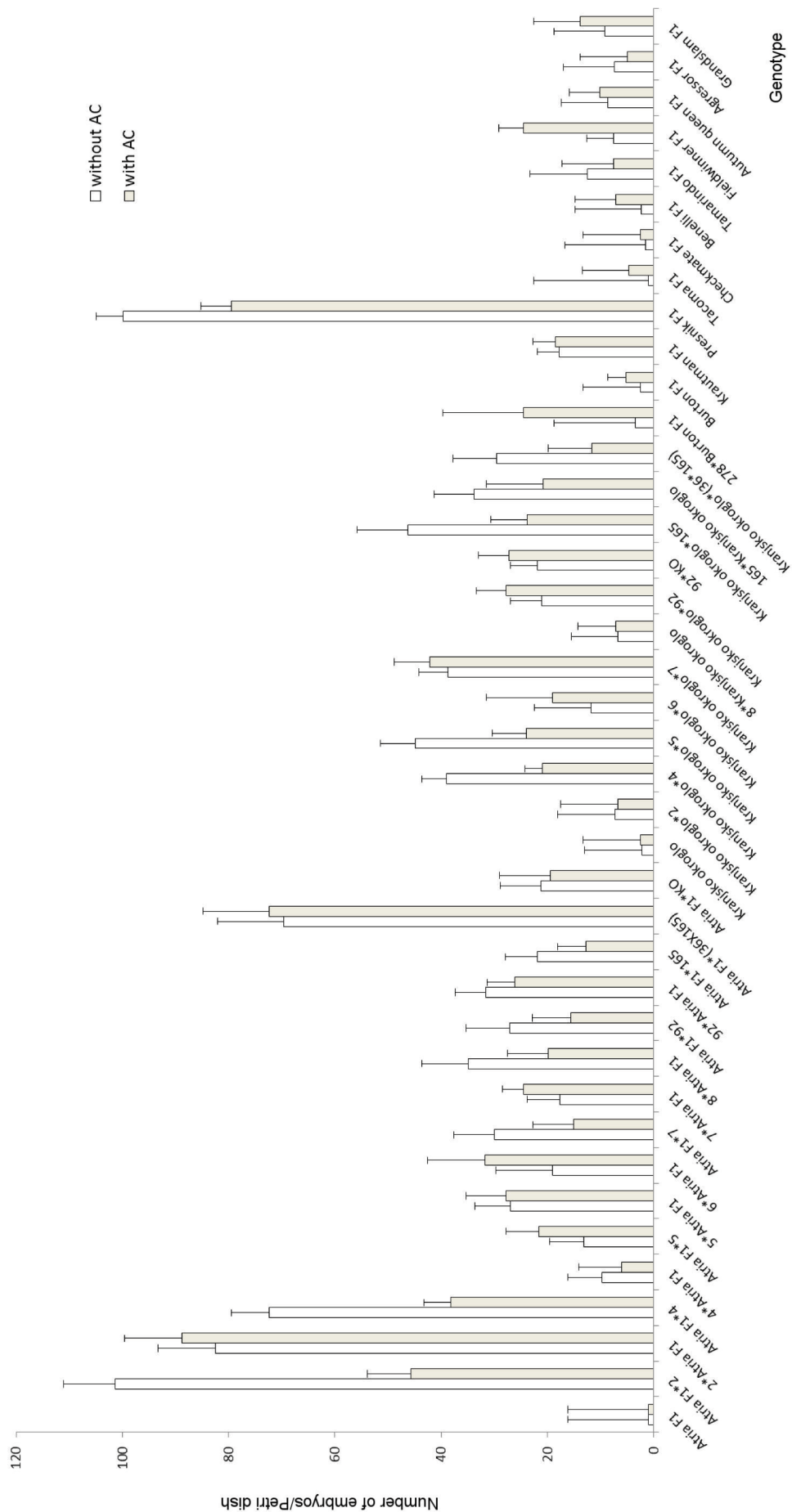


Figure 2. Results of microspore embryogenesis in white cabbage genotypes: average number of embryos formed per Petri dish for 40 genotypes with and without activated charcoal (AC) treatment (total number of embryos was 20,032). Whisker bar (only in positive direction shown) represents standard error

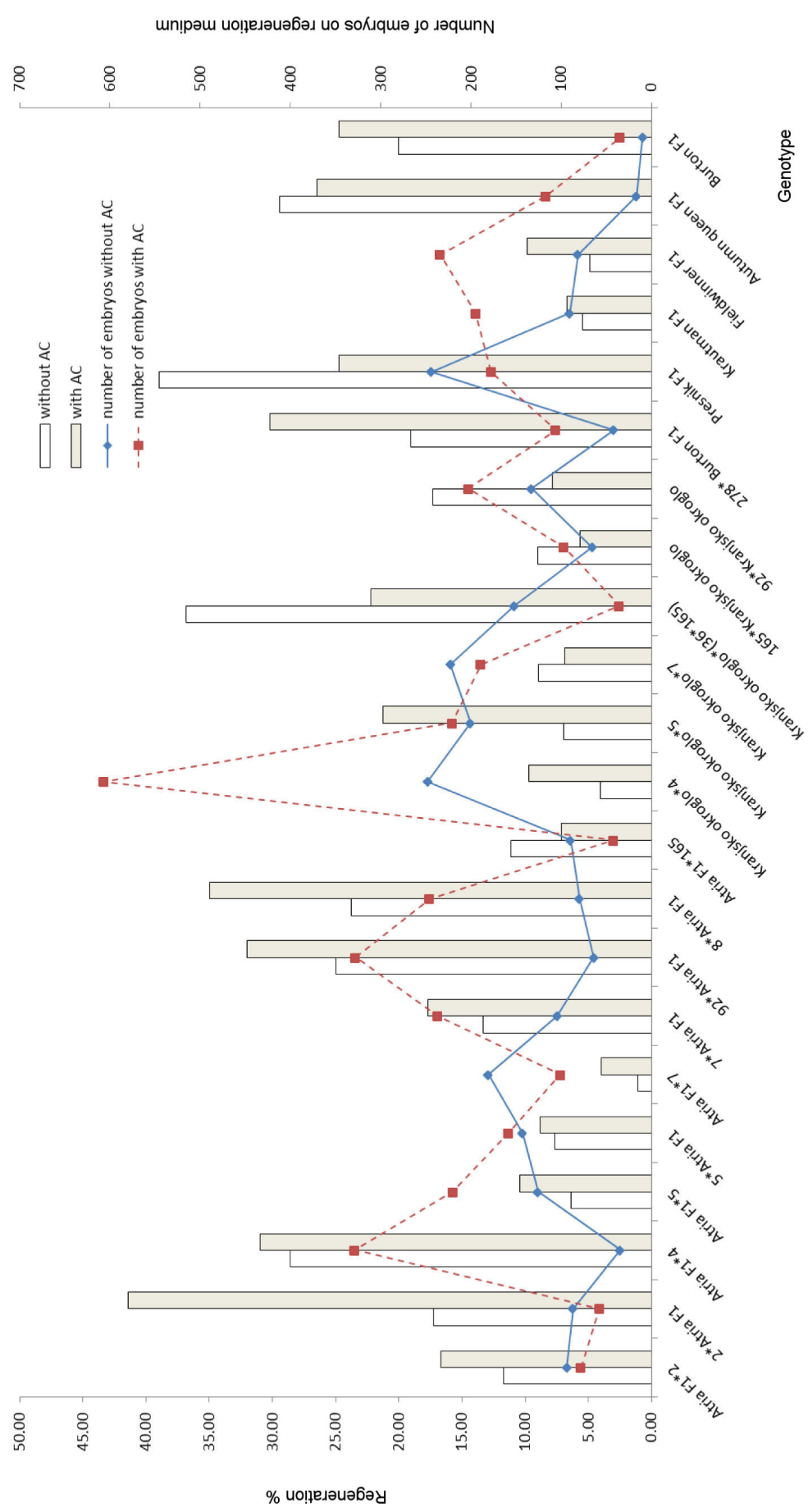


Figure 3. White cabbage embryos formed by microspore culture placed directly onto regeneration media. Efficiency of germination of embryos originated from induction treatments with and without activated charcoal (AC) treatment for 22 genotypes scored after 30 days on germination medium. Lines: numbers of inoculated embryos; bars: percentage of regeneration

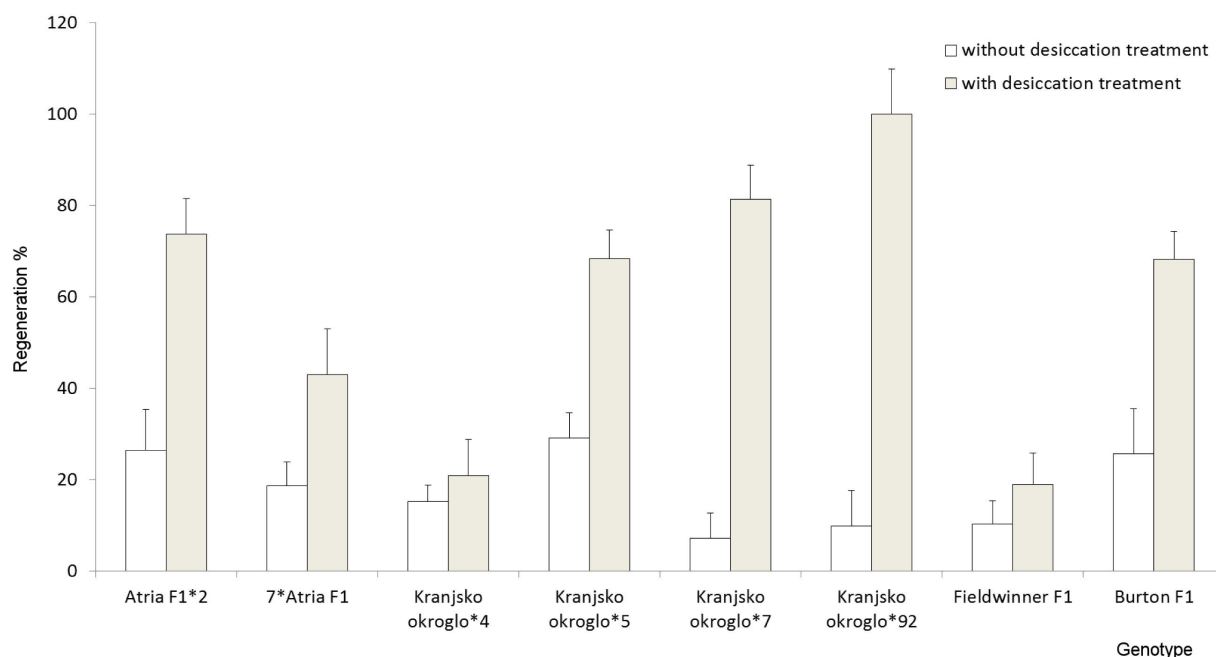


Figure 4. White cabbage embryos formed by microspore culture placed directly onto regeneration media or first treated with ABA and desiccated for 4 weeks. Efficiency of germination for abscisic acid-treated embryos (2,097) for eight genotypes after 30 days of culture on germination medium. Whisker bar (only in positive direction shown) represents standard error

responsive genotypes. On average, the haploid induction response of low-responsive cultivars ('Burton' F₁, 'Atria' F₁, K. O.) was 7.0 embryos/Petri dish, but the average of these hybrids crossed to responsive DH lines was 26.6 embryos/Petri dish. The most responsive genotypes were 'Presnik' F₁ (99.8/Petri dish without AC, 79.4/Petri dish with AC), 'Atria × 2' (101.4/Petri dish without AC, 45.7/Petri dish with AC), '2 × Atria' (82.5/Petri dish without AC, 88.7/Petri dish with AC), 'Atria × 4' (72.3/Petri dish without AC, 38.2/Petri dish with AC) and 'Atria × (36 × 165)' (69.6/Petri dish control, 72.3/Petri dish with AC). The genotype/treatment interaction was evident from the various effects of AC addition, with regard to genotype. The addition of AC to the induction media resulted in increased embryo formation in several low-responsive genotypes, such as 'Fieldwinner' F₁ and '278 × Burton' F₁, but its effect on embryo formation was predominantly negative for high-responsive genotypes. Reciprocal effects were tested by crossing 'Atria' F₁ and 'Kranjsko okroglo' in both directions. In one case, the 'Atria × 4' and '4 × Atria' effect was noted, as those having 'Atria' F₁ as the female component were considerably more responsive to both treatments and vice versa, but in the other six crosses, the differences were minimal (Fig. 2).

Embryos formed in the induction experiment were not of equal size, with those regenerated on

media containing AC appearing larger than their non-AC media counterparts (Fig. 1A). Therefore, we analysed their potential for further development. As described in the Material and Methods section above, two procedures were used to achieve the regeneration into plantlets.

With the first procedure, 2,514 embryos obtained without the addition of AC were transferred directly to the regeneration medium without desiccation treatment. In 16/22 genotypes, regeneration of embryos was higher when obtained from induction media containing AC (Fig. 3) compared to those without AC, but the differences were not statistically significant. Overall, regeneration of embryos by this procedure was 18.2%. Regeneration of plantlets was also highly influenced by genotype, while no apparent correlation existed between embryo induction responsiveness and plantlet formation. Plantlets obtained by this method often exhibit abnormal subsequent development or hyperhydricity (Fig. 1B).

With the second procedure, 4,016 embryos obtained with the addition of AC were either placed on media without desiccation or treated with ABA and desiccated. In all genotypes, desiccation treatment highly improved plantlet development, reaching 57.6% overall (Fig. 4) and, furthermore, no abnormal formation or hyperhydricity were noted (Fig. 1C, 1D).

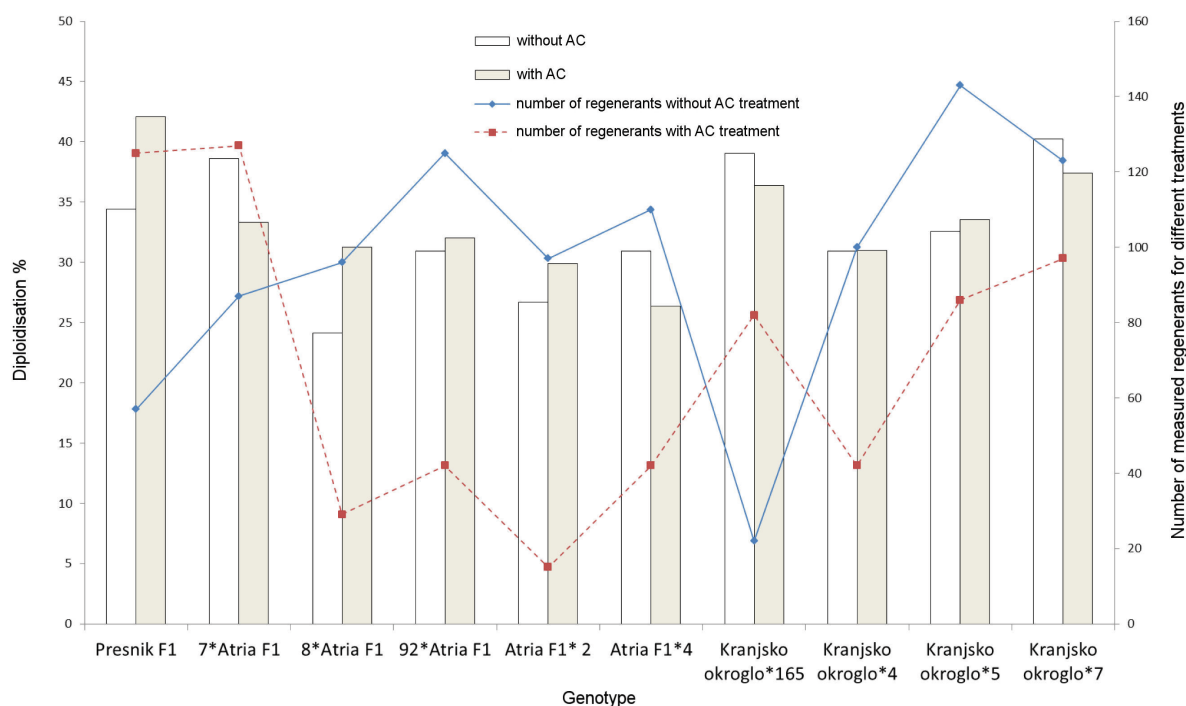


Figure 5. Diploidisation efficiency as a percentage of diploid plants of white cabbage for 10 high-yielding genotypes (AC, activated charcoal). Bars: percentage of diploidized plantlets originated from embryos induced with or without addition of AC. Lines: numbers of plantlets measured for ploidy

Data on ploidy level were collected to investigate whether embryos originated on media with or without AC respond differently (Fig. 5). In total, 2,033 plants were measured, of which 42.5% were diploid and the rest being haploid (50.8%), triploid (0.6%), tetraploid (5.8%), pentaploid (0.1%) and octoploid (0.2%). The percentage of spontaneous diploidisation was very similar between the two procedures, indicating a minimal influence of induction treatment on the proportion of spontaneous diploids or other classes of ploidy.

DISCUSSION

The embryo induction experiment with a large number of genotypes demonstrated that the tested commercial varieties/hybrids tend to possess a low level of embryogenic potential. The exception was the hybrid variety ‘Presnik’ F₁, which was bred by Biotechnical Faculty of Ljubljana and was itself a cross between two highly responsive DH lines. Improvement of regeneration achieved by crossing with a responsive line, as was also noted in our previous work (Rudolf et al., 1999), was confirmed in all tested genotypes. The reciprocal effect was examined for the first time. The finding that one of seven combinations was influenced by the maternal effect (low-responding hybrid being superior as the female partner) might be a coincidence or an interesting outcome that requires further attention.

The addition of AC to the induction media has a noted effect on embryos, but lesser, for instance, than that reported in other species, like rocket (Leskovšek et al., 2008). Increased embryogenic capacity has also been found in broccoli (*B. oleracea* var. *italica*) (Haeyoung et al., 2011). A very similar response has been observed in Chinese cabbage (*Brassica campestris* spp. *pekinensis*) (Han et al., 2014), where the addition of AC increased plantlet regeneration. In several subspecies of *Brassica rapa*, further development of obtained microspore-derived embryos can be approached in different ways (Takahashi, 2012). The cited authors noted that the positive effects of cytokinin were cancelled by the addition of AC. Moreover, our results presented in the current study on white cabbage showed for the first time that AC exerted a pronounced influence on embryo size and, therefore, affected further development of the embryos.

Haddadi et al. (2008) reported in rapeseed (*B. napus*) a rather negative effect of desiccation, with either no desiccation or 40 µg ABA treatment followed by 5 minute desiccation being optimal. The presented experiments showed that, in white cabbage, using direct placement on media without ABA-induced desiccation reduces survival to a large extent, while careful ABA-induced desiccation can be performed efficiently when carefully designed and carried out.

An increase in spontaneous ploidy level is frequent in microspore-derived plantlets of brassicas and, consequently, doubling treatments are infrequent. For instance, Takahashi et al. (2012) reported that in *B. rapa* spontaneous doubling reached 66-100%. Our results also showed sufficient spontaneous doubling, but no influence of AC addition in the induction media.

CONCLUSIONS

1. When commercial varieties are used as parents, it is advisable to use at least one responsive parent crossed to non-selected genotypes to increase the haploid regeneration frequency.
2. Responsive parents can be used as the male or female component.
3. AC should be included in the induction media, given that somewhat larger and well-formed embryos capable of regeneration can be expected, relative to induction in non-AC media.
4. ABA-induced desiccation should be applied, considering that the conversion of embryos into plantlets is highly improved (up to 57.6%).
5. Expected spontaneous chromosome doubling is not affected by the presence of AC in the induction media and the proportion reached (42.5%) is considered sufficient for breeding programmes.

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AUTHOR CONTRIBUTIONS

K.R.P. – designed the experiments and conducted the statistical analysis; U.K.P. – performed parts of the experiments; B.B. – helped in ploidy measurements and wrote the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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