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Diversity of diploid androgenic Brussels sprout plants of R_0 and R_1 generations

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Abstract. Androgenic Brussels sprout plants were produced by the use of anther culture from the donor cultivar ‘Philemon F_1’. A total of 96 plants obtained from 20 androgenic R_0 genotypes assigned as diploids were evaluated both in the generative and vegetative stage, in respect of their morphological characters: mean plant height; leaf size, colour and waxiness; leaf blade shape, blistering and attitude; number of sprouts; as well as their self-incompatibility and fertility. Androgenic R_0 plants derived from each of the 20 embryos were highly diversified and differed from the donor in one or more morphological traits in the vegetative stage. Evaluated populations also varied in fertility and self-incompatibility. Six androgenic genotypes that set a sufficient amount of seeds of the R_1 generation and ‘Philemon F_1’ were evaluated in the field in respect of plant height, total and marketable yield per plot, shape of stem with sprouts, shape and density of sprouts, and spacing between sprouts. Only four diploid R_0 and R_1 populations may have some value for further breeding, as they are characterised by good vigour, high or medium ability for sprout generation, and sufficient fertility.

Key words: Brussels sprouts, diploid R_0 and R_1 generation, fertility, morphological traits, self-incompatibility.

Introduction

Androgenesis is mainly used for generating doubled haploid (DH) homozygous lines as potential parental components for producing F_1 hybrids. Obtaining and evaluation of androgenic plants of *Brassica* crops – such as cabbage, broccoli, cauliflower and Brussels sprouts – was described by many authors (Chiang et al. 1985; Ockendon 1986, 1988; Dore and Boulidard 1988; Chauvin et al. 1993; Górecka et al. 1997; Farnham 1998; Kamiński et al. 1999, 2004; Wang et al. 1999) and proved that anther and microspore culture could be effective and useful for their breeding. An advantage of anther and microspore culture over the traditional breeding methods for cabbage crops consists in shortening of the breeding cycle by skipping steps of inbreeding for several generations. Androgenic plants from *B. oleracea* crops derived by the use of anther culture usually contain a mixture of haploids, diploids, triploids, tetraploids, octoploids and aneuploid individuals (Chiang et al. 1985; Ockendon 1988; Chauvin et al. 1993; Farnham 1998) due to spontaneous and irregular polyploidisation during the culture. Diploid individuals, the most desired for breeding, should be identified among the mixtures of regenerants and used as potential homozygous DH lines in hybrid combinations (Farnham 1998; Wang et al. 1999). The number of diploid plants among androgenic populations of Brussels sprouts usually ranged from about 30% (Ockendon 1986) to more than 50% (Kamiński et al. 2004), which is also characteristic for other *B. oleracea* crops, as described by Keller and Armstrong (1983); Chiang et al. (1985); Górecka (1998); Farnham (1998); Kamiński (1999).

Large-scale production of pure breeding lines as DH generations with the method mentioned above is justified only if embryos are produced at a reasonable frequency and when androgenic plants obtained are either 1n or 2n and arise as

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spontaneously doubled haploids (Ockendon 1988). Androgenic cabbage populations derived by anther culture usually contain a mixture of plants with unknown commercial value and diversified morphological traits (Kamiński et al. 1999, 2004). Brussels sprouts, like other cabbage plants, is characterised by biennial habit, strong inbreeding depression, long vernalization period, and usually exhibits diversified and/or high level of self-incompatibility (Ockendon 1973; Wallace 1979; Hoser-Krauze 1993). For breeding purposes, androgenic DH lines of Brussels sprouts should fulfil several requirements, such as desired morphological traits, high level of self-incompatibility, high seeding index, good combining ability, and genetic stability in consecutive generations. Obtaining seeds of DH (R_1) populations is one of the most critical tasks, as DH lines usually give very low seed yields in comparison with the lines derived from classic selection methods (Chauvin et al. 1993; Farnham 1998).

The aim of this study was to evaluate diploid androgenic R_0 and R_1 populations of Brussels sprout plants obtained from the commercial hybrid cultivar 'Philemon F_1' in both the vegetative and generative stage, in respect of several morphological and agronomic traits, fertility and self-incompatibility, to assess their value for breeding.

Material and methods

Plant material and evaluation of morphological traits

Androgenic Brussels sprout plants of the R_0 generation were obtained from the hybrid cultivar 'Philemon F_1' by the use of anther culture at the Research Institute of Vegetable Crops, Skierniewice, Poland, in 2001. From the total of 174 androgenic R_0 plants, 96 individuals (55.2%) derived from 20 embryos were assigned as diploids by the use of chromosome count and flow-cytometry analysis (Kamiński et al. 2004).

The plants were grown in 5-litre plastic pots containing Kronen Mix and placed on the ground in an uncovered tunnel house. Fertilisation, watering and plant protection against pests and diseases was provided according to current recommendations for that species. Evaluation of morphological traits of the diploid Brussels sprout R_0 generation was performed separately for all plants obtained from each of 20 embryos in comparison to the donor cultivar 'Philemon F_1'. In October 2001, when plant growth ended, diploid androgenic Brussels sprout plants and the donor cultivar were evaluated in the vegetative stage in respect of mean plant height (cm) and ability for developing sprouts. Leaf size, colour, waxiness, shape, blistering and attitude of the leaf blade, were also evaluated basing on the observation of 4–5 leaves taken from the middle part of the stem of each plant. The morphological traits were classified on the multigrade scale of the International Board for Plant Genetic Resources (IBPGR) (see the footnotes in Table 1).

Pollination, fertilisation and seed setting

From November 2001 to the end of February 2002 all diploid androgenic plants of Brussels sprouts and 'Philemon F_1' were vernalised at 6°C under natural day length to induce the generative stage. Seed stalks were isolated a few days before tests, with the covers made of transparent paper to avoid undesired cross-pollination by insects. Pollination was performed in two replications on 7–10 green buds and freshly developed flowers from a single stalk of each genotype in a greenhouse during the growing season, from March to May 2002. Plants were self-pollinated both in the green bud stage to obtain seeds and at the open flower stage to assess their self-incompatibility. The level of self-incompatibility was measured by two methods: pollen tube penetration after self-pollination and seed set counts. Twenty-four hours after pollination, pollen germination and pollen tube growth were measured in 3–5 green buds and flowers. The staining of pollinated pistils with aniline blue (Martin 1959), modified by Dyki (1978), was originally used to estimate incompatibility of cauliflower plants. In our study the pistils were macerated in 1% NaOH, stained with aniline blue, smashed in glycerine, and observed under a microscope NU-1 Zeiss with HBO lamps and OG 1 and BG 3 filters at 125 or 250 magnification. In such conditions, typical yellow fluorescence of callose present in pollen grains and pollen tubes was observed. Cytological symptoms of incompatibility were compared with compatibility symptoms between pollen and stigma. In the second method, all pollinated siliquas were harvested 70–90 days after pollination, when they reached maturity. Dried siliquas were counted, and fully developed seeds were extracted separately for each genotype. Androgenic plants that showed good pollen tube

Table 1. Morphological characters of diploid Brussels sprout androgenic R_0 plants from 20 embryos in the vegetative stage, and in the donor cultivar ‘Philemon F_1’

Cultivar/ embryo code	No. of plants per embryo	Mean plant height (cm)	Leaf		Waxiness[3]	Leaf blade			No. of sprouts[7]
			size[1]	colour[2]		shape[4]	blistering[5]	attitude[6]	
Philemon F_1		34	3	3	3	1	3	5	7
P1	9	27.7	3	3	3	1	3	7	5
P2	1	12	1	3	3	1	3	5	3
P3	6	23.7	3	5	3	1	5	5	3
P4	9	24.6	1	3	5	1	3	3	5
P5	9	63	1	3	3	1	5	3	7
P6	2	25	3	3	5	1	3	3	3
P7	7	28	3	5	3	1	3	7	7
P8	1	28	1	1	1	1	7	5	3
P9	2	32.5	3	3	3	1	5	5	5
P10	5	18.4	1	3	5	1	5	3	5
P11	6	25.8	3	5	5	1	3	5	7
P12	3	31.7	1	5	5	1	3	5	7
P13	1	37	3	3	5	1	3	5	7
P14	8	45	3	5	5	1	3	5	5
P15	6	26	1	3	3	3	3	3	1
P16	1	34	3	3	5	1	3	5	7
P17	1	46	1	3	5	1	0	3	3
P18	12	33.3	3	3	5	1	3	5	7
P19	6	36.2	3	3	5	3	3	3	3
P20	1	33	1	5	3	1	3	5	7

[1] Leaf size:1 = small 3 = medium 5 = large
[2] Leaf colour: 1 = light green 3 = green 5 = dark green
[3] Waxiness:1 = light 3 = medium 5 = strong
[4] Leaf blade shape: 1 = orbicular 3 = elliptic
[5] Leaf blade blistering: 0 = none 3 = low 5 = intermediate 7 = high
[6] Leaf blade attitude: 3 = convex 5 = straight 7 = concave, dropping
[7] Number of sprouts: 1 = none 3 = single 5 = medium number 7 = plenty

penetration and development of at least one seed per siliqua when pollinated at the opened flower stage were classified as self-compatible. Plants that did not set seed when pollinated at the open flower stage and with absence or only single pollen tubes penetrating the style were considered self-incompatible.

Evaluation of the R_1 generation

Androgenic Brussels sprout R_1 lines were obtained by self-pollination of R_0 genotypes. Six androgenic genotypes (P3, P4, P5, P11, P14 and P18), which set a sufficient amount of seeds of the R_1 generation, and ‘Philemon F_1’ were evaluated in the field in the Research Institute of Vegetable Crops, Skierniewice, in 2003. The soil type was a pseudopodsol over loamy sand (1.5% organic matter, pH 6.5). The tested plants developed from seeds in the greenhouse in mid-April. One-month-old seedlings were planted in the field (spacing 50 cm × 60 cm) in a completely randomised block design with three replications. Each plot consisted of ten plants in one row. Fertilisation, pest and disease control followed the current recommendations. Plants were harvested in mid-November, when sprouts reached maturity. Plant height, and total and marketable yield per plot were measured. Results were elaborated statistically by an analysis of variance (ANOVA). The significance of differences among means was evaluated by Newman-Keul’s test at $\alpha = 0.05$. The shape of stem with sprouts, shape and density of sprouts, and spacing between them were classified separately for each plot by the use of the multi- grade IBPGR scale.

Results

Morphological variation

Numbers of diploid androgenic Brussels sprout plants of the R_0 generation obtained from the hybrid cultivar ‘Philemon F_1’ varied between the 20 lines (Table 1). For six embryos (P2, P8, P13, P16, P17, P20) only single R_0 plants were obtained, while the highest number (12) of diploid R_0 plants was scored for embryo P18. The mean number of androgenic diploid plants regenerated from a single embryo was 4.8. No evident morphological differences among androgenic plants derived from a single embryo were observed, but populations obtained from different embryos were highly diversified in respect of all tested traits. Mean plant height ranged from 12 cm for R_0 plants derived from embryo P2 to 63 cm for those from embryo P5 (Table 1, Figure 1). The mean for all tested diploids (32.7 cm) did not differ significantly from ‘Philemon F_1’ (34 cm). Two types of leaf size were observed among the tested diploid R_0 Brussels sprout populations: small (45%) and medium (55%), the latter typical for the donor cultivar. The offspring of 13 embryos had green leaves, typical for ‘Philemon F_1’; 6 of them were dark-green and only one plant from embryo P8 had light green leaves. Most of diploid androgenic populations (55%) expressed stronger waxiness than the donor cultivar. Only two generations obtained from embryos P15 and P19 had the atypical elliptic shape of leaf blades, while the other embryos had orbicular leaves and showed high similarity to the donor variety (Figure 1). A stronger diversity was observed among diploid androgenic generations in leaf blade blistering and attitude (Table 1, Figure 1). The majority (70%) of diploid genotypes had the typical low blistering, but four of them (P3, P5, P10, P11) were at the intermediate level and single genotypes had none (P17) or high (P8) leaf blade blistering. Six genotypes (55%) were characterised by the typical, straight attitude of leaf blades, while the others were convex (10%) or concave (35%). Only eight genotypes (P5, P7, P11, P12, P13, P16, P18, P20) formed plenty

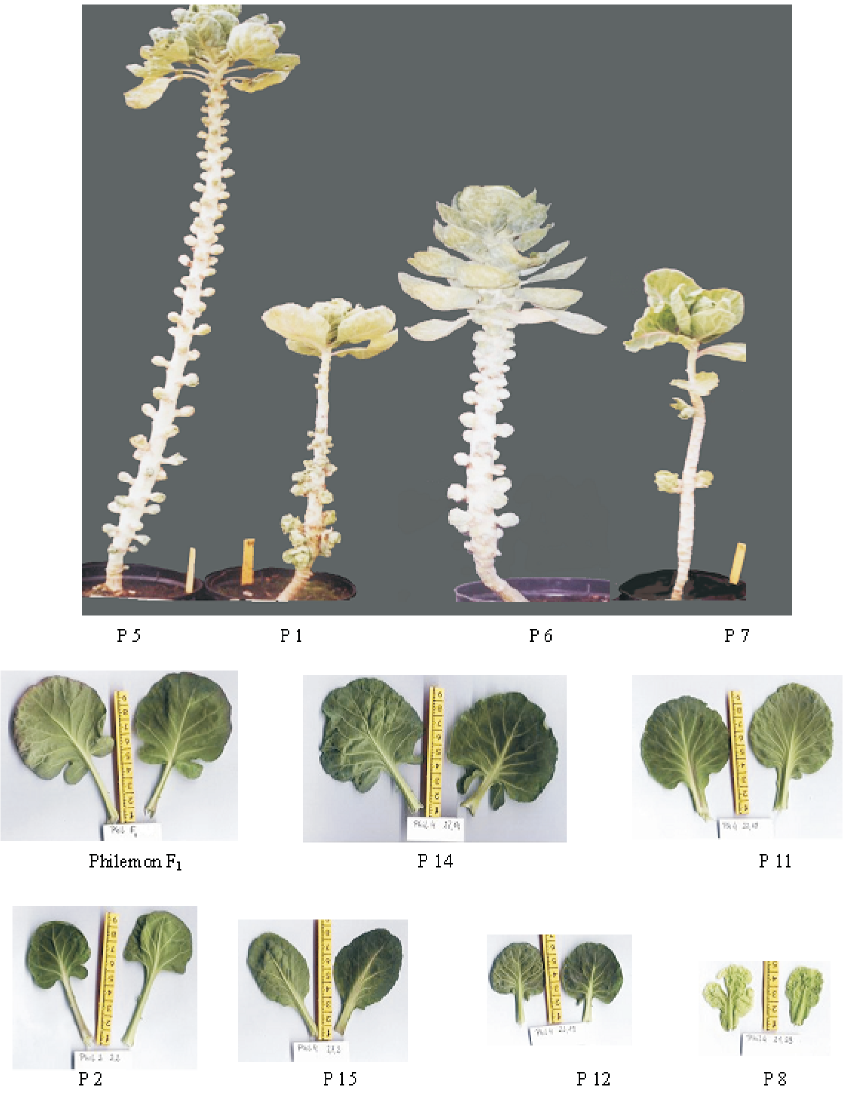

Figure 1. Examples of morphological differences in plant height, sprout quantity and leaf morphology of Brussels sprout diploid R_0 androgenic plants derived from different embryos

sprouts, which is typical for their donor cultivar. Genotype P15 had no sprouts, six genotypes (P2, P3, P6, P8, P17, P19) exhibited only single and deformed sprouts, and five others had a medium number of sprouts (P1, P4, P10, P11, P14).

Fertility and self-incompatibility

Androgenic diploid plants varied also in fertility and self-incompatibility level when evaluated by both the staining method and seed counts (Table 2). Pollen grains were visible on stigmas of a majority of pistils (Figure 2a). Only for plants derived from three embryos (P13, P16, P17) pollen on stigmas was not found after pollination in the bud as well as in the open flower stage. The fluorescence of stigma papillae was often much stronger than fluorescence of pollen grains and was observed even if there were no pollen grains on the stigma (Figure 2b). Pollen germinated more frequently after the bud pollination than at the open flower pollination, where short, burst and cracked pollen tubes dominated (Figure 2c). They penetrated only stigma tissue and did not reach the style and ovary (Figure 2d). In plants with this type of pollination both in buds and open flowers, generally no seed set was observed. In pistils of ‘Philemon F_1’ and in plants derived from eight embryos P2, P3, P4, P5, P6, P11, P14, P18, long pollen tubes reached ovules in large (Figure 2e) or small numbers (Figure 2f). The presence of long pollen tubes in ovaries was the proof of fertilisation process, which initiated seed setting. Only twelve genotypes set seed when pollinated in the green bud stage. The number of seeds per siliqua ranged from 0.41 for P8 to 6.9 for P11 and 6.7 for P5. In our study three genotypes showed some level of self-compatibility (P6, P11

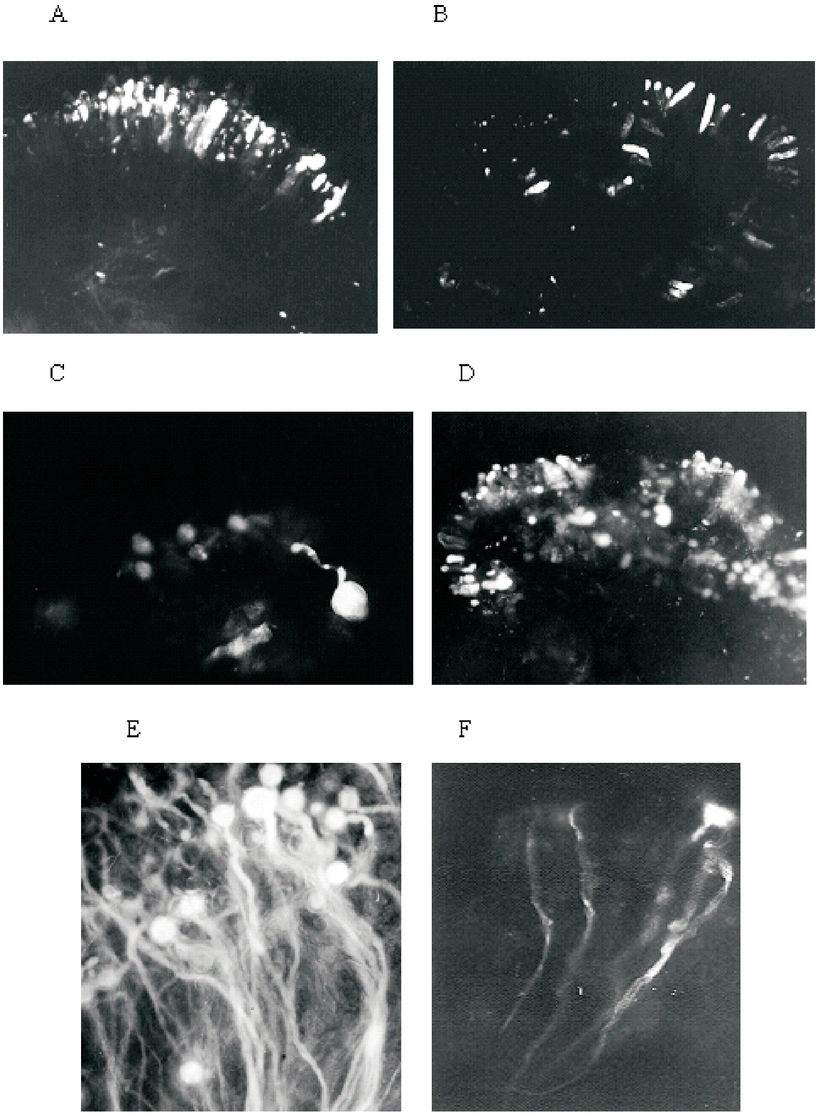

Figure 2. Pollen-pistil interactions of diploid androgenic Brussels sprout plants visible in fluorescence light under a microscope. A, B, C, D = inhibition of pollen tube growth. E, F = pollen tube growth during fertilisation

Table 2. Pollination, fertilisation and seed setting in diploid Brussels sprout androgenic R_0 plants from 20 embryos and in the donor cultivar 'Philemon F_1'

Cultivar/ embryo code	Pollination*/fertilisation**		No. of seeds per siliqua	
	bud pollination	open flower pollination	bud pollination	open flower pollination
Philemon F_1	3/3	1/0	15	0
P1	1/0	1/0	0	0
P2	2/1	3/1	0.43	0.10
P3	3/1	1/0	2.50	0
P4	3/1	2/0	3.75	0
P5	3/3	1/0	6.67	0
P6	3/1	3/3	2.42	4.50
P7	2/0	1/0	0	0
P8	1/0	2/0	0.41	0
P9	1/0	2/0	0	0
P10	2/0	1/0	0	0
P11	2/2	3/1	6.93	1.30
P12	2/0	2/0	0	0
P13	0/0	0/0	0	0
P14	2/1	3/0	2.1	0
P15	2/0	3/0	0	0
P16	0/0	0/0	0	0
P17	0/0	0/0	1	0
P18	3/1	3/0	1.21	0
P19	3/0	3/0	0.50	0
P20	1/0	2/0	0	0

* Number of pollen grains on stigma: 0 = absent, 1= single, 2 = quite a few, 3 = plenty
** Number of pollen tubes in pistil: 0 = absent, 1= single, 2 = quite a few, 3 = plenty

and P2) as they set seed both in the bud and open flower stages (from 0.1 to 4.5 seeds per siliqua) while the donor 'Philemon F_1' and other genotypes (P3, P4, P5, P8, P14, P18, P19), which set seeds only when pollinated in the green bud stage, were strongly self-incompatible. All of the tested androgenic genotypes set fewer seeds per siliqua than 'Philemon F_1' when pollinated in the bud stage (Table 2).

R_1 generation

The androgenic R_1 Brussels sprout generation varied in all traits tested in the field (Table 3). Four lines (P3, P11, P14 and P18) had the same mean height of plants as 'Philemon F_1' (76 cm), while P4 (62.9 cm) was significantly lower and the P5 R_1 generation (87.1 cm) was higher than the donor cultivar. Total yield of sprouts per plot ranged from 1.4 kg for the P3 R_1 line to 7.0 kg for P5 and P11. 'Philemon F_1' had a higher total (8.0 kg) yield of sprouts than all other tested genotypes. Marketable yield of sprouts for 'Philemon F_1' (6.7 kg) did not differ significantly from those of P5 (5.3 kg) and P11 (5.2 kg) androgenic R_1 lines. The P3 androgenic line had the lowest marketable (0.2 kg) yield per plot as well as the loosest sprouts and largest spacing between them. For almost all R_1 populations a cylindrical shape of stem, typical for the donor cultivar, was observed. Only the P11 line had a conical shape of stem with sprouts. The P18 line had untypical broad obovate sprouts, while those of all other genotypes were circular. The P14 androgenic R_1 generation had sprout density as high as that of 'Philemon F_1' but most other lines were characterised by medium density of

Table 3. Mean plant height, mean total and marketable yield of sprouts per plot (kg), shape of stem with sprouts, sprout shape and density, and spacing between sprouts in the diploid Brussels sprout androgenic R_1 generation obtained from 6 embryos, and in the donor cultivar 'Philemon F_1'

Cultivar/ embryo code	Mean plant height (cm)	Total yield per plot (kg)	Marketable yield per plot (kg)	Shape of stem with sprouts[1]	Sprout		
					shape[2]	density[3]	spacing[4]
Philemon F_1	76.0 b	8.0 d	6.7 c	2	1	3	1
P3	73.9 ab	1.4 a	0.2 a	2	1	1	3
P4	62.9 a	5.4 bc	3.4 b	2	1	2	1
P5	87.1 c	7.0 cd	5.3 c	2	1	2	2
P11	70.9 ab	7.0 cd	5.2 c	1	1	2	2
P14	70.6 ab	4.2 b	1.8 b	2	1	3	2
P18	67.9 ab	3.8 b	3.1 b	2	2	2	2

Values followed by the same letters did not differ significantly at $\alpha = 0.05$ (Newman–Keul's test)
[1] Shape of stem with sprouts:1 = conical, 2 = cylindrical
[2] Sprout shape: 1 = circular, 2 = broad obovate
[3] Sprout density: 1 = loose, 2 = medium, 3 = dense
[4] Sprout spacing: 1 = small, 2 = medium, 3 = large

sprouts. The smallest spacing between sprouts was observed for 'Philemon F_1' and the P4 androgenic line.

Discussion

'Philemon F_1' is one of the most efficient donor cultivars, as it has the highest embryogenic potential described by Krzyżanowska and Górecka (2004). Production of a large population of diploid androgenic R_0 plants (Kamiński et al. 2004) proved that the anther culture method used for this cultivar was effective. Generally, none of the 20 diploid androgenic R_0 generations had all traits typical for 'Philemon F_1' in the vegetative stage. Three R_0 generations (P6, P13 and P16) differed from their donor in only one of eight traits tested. By contrast, diploid androgenic R_0 plants derived from 3 other embryos (P10, P17 and P8) differed from 'Philemon F_1' in most of their morphological characters. Such a diversity in androgenic populations, in comparison to their donors, has mostly a genetic background. As a result of regeneration and differentiation during anther culture, populations obtained from different embryos may contain unique gene combinations (Niemirowicz-Szczytt 1997). The lack of natural selection among R_0 androgenic plants of Brussels sprouts, in comparison to the traditional breeding, would probably have a negative influence on the quality of the obtained material when inferior lines would not be rejected at the early stage of breeding. Growth conditions for 'Philemon F_1' and the tested diploid R_0 generation at this evaluation differed from those in open field experiments and caused probably the smaller size of all plants irrespective of genotype. However, observations of morphological traits in the R_0 generation enabled the very early selection of diploid genotypes with the best genetic potential. Eight genotypes (P5, P7, P11, P12, P13, P16, P18, P20), which created abundant sprouts, may have some value in further breeding programmes, and the other 12 genotypes with a lower number of sprouts seem to be out of interest.

The differences in seed productivity and self-incompatibility between the tested genotypes can be attributed to the genetic background of the R_0 plants obtained from different embryos of 'Philemon F_1' and – to some extent – to environmental factors, such as temperature during the growing period. The high self-incompatibility level of most of the tested diploid R_0 generations can be advantageous, as it will minimise the possibility of obtaining undesired sibs in hybrid production. Brussels sprout inbreed lines described by Ockendon (1973) showed a strong plant-to-plant variation in the level of self-incompatibility during the flowering season. To examine the utility of ten fertile androgenic genotypes of Brussels sprouts, further investigations should be performed after R_0 generation both in the generative stage and in the field experiments in the vegetative stage. Because of the high variability caused by environmental factors, repeated tests are essential, as the plants that initially appear to be highly self-incompatible may show a marked degree of self-compatibility afterwards (Chauvin et al. 1993; Farnham 1998). Irrespective of the normal flower morphology and the presence of abundant pollen in all diploid generations of R_0 Brussels sprout plants, half of genotypes set seed neither at the bud nor at the open flower stage. Ten genotypes that showed a very low level of fertility cannot be used for further breeding. Our observations are in accordance with Chauvin et al. (1993), who observed poor seed production in DH broccoli, compared with the lines derived from classic selection methods, and concluded that it represented a severe limitation to the value of DH plants as potential parents for F_1 hybrids. Microscopic analyses showed that information exchange between pollen and pollinated pistil in diploid androgenic Brussels sprout plants took place on the surface of stigma or inside its tissue. The incompatibility of pollen grains with stigma was determined on the basis of weak adhesion, germination disturbances, and callose synthesis in tips of short pollen tubes and in stigma cells that were in direct contact with pollen grains. Such reactions are characteristic for plants with 3-cell pollen grains and dry surface of the pistil and they were typical examples of sporophytic self-incompatibility in *Brassica oleracea* (Gaude and Dumas 1987; Nasrallah and Nasrallah 1993; Stephenson et al. 1997; Takayama and Isogai 2003) and other *Brassica* species (Kandasamy et al. 1989; Schopfer et al. 1999; Hatakeyama et al. 2001).

Our observations of morphological and cytological characters among diploid androgenic R_0 generations of Brussels sprouts indicate that anther culture provides some opportunity to select desired genotypes. However, only five populations (P4, P5, P11, P14 and P18) had both good vigour, high or medium ability for sprout creation, and sufficient seed numbers if pollinated at the bud stage. Seeds of the R_1 DH generation of those genotypes were assigned for further evaluation under field conditions to obtain additional information on their agronomic traits and for selecting useful androgenic individuals for the breeding. The other 15 diploid androgenic generations (75% of the whole examined diploid population), because of their insufficient fertility, low sprout abundance or weak vigour, seem to be useless for further breeding. The low level of self-incompatibility of the P11 line may be also a disadvantageous trait, which may decrease its value for breeders.

Taking into consideration that the total number of 174 androgenic Brussels sprout plants was obtained from 38 embryos (Kamiński et al. 2004), and that after all selections in the R_0 generation, only five (13.2%) androgenic diploid generations may have some value for breeding, the practical effectiveness of the anther culture method seems to be relatively low.

Field evaluation of six R_1 androgenic lines in comparison to the donor cultivar 'Philemon F_1' proved that the diversity between them was as high as that observed in the R_0 generation. Four lines (P4, P5, P11 and P18) were characterised by a relatively high marketable yield of sprouts per plot and can be used for further breeding. Line P3, with the lowest yield of sprouts and undesired morphological characters, was assessed as useless for breeding both in R_0 and R_1 generations. The lower yield of sprouts for all androgenic lines than for 'Philemon F_1' may be caused by a depression typical for inbreed lines. Although plants of all six R_1 generations seemed to be uniform as they were evaluated according to morphological and agronomic characters, further investigations – including molecular and isozyme analysis – will be performed to assess their level of homozygosity.

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