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Evaluation of the propensity of interspecific hybridization between oilseed rape (*Brassica napus* L.) to wild-growing black mustard (*Brassica nigra* L.) displaying mixoploidy

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Highlights

- Significant interspecific hybridization in *B. nigra* (♀) x *B. napus* (♂) F1 hybrids
- Gene transfer evident in F1 hybrids under both controlled and open-field conditions
- Mixoploidy in wild-growing *B. nigra* increases hybridization propensity with *B. napus*
- Has gene flow from *B. napus* x *B. nigra* been underestimated in the environment so far?

A B S T R A C T

Potential gene flow from transgenic *Brassica napus* to widely-distributed, cross-compatible weedy relatives has received significant attention. All previous, albeit scarce, research has shown little to no success in producing viable F1 hybrids between *B. napus* (n= 38) and *B. nigra* (n= 16). The present study tested the working premise that the propensity for interspecific hybridization is significantly higher between *B. napus* and wild-growing, *B. nigra* displaying mixoploidy (n= 32). Controlled hybridization was performed using local, wild-growing *B. nigra* (♀) x transgenic (*Bt Cry1Ac*) *B. napus* (♂). Spontaneous hybridization was performed using the same *B. nigra* (♀) population x non-transgenic *B. napus* (♂) under sympatric open-field and greenhouse conditions. The total hybridization frequency, determined by the functional expression of the *Bt* Cry1Ac endotoxin, was

1.8 % of the F1 hybrids (n= 35). Gene flow from non-transgenic *B. napus* to *B. nigra* ranged from 4 to 29 % in F1 hybrids, with combined wind- and wild-insect-mediated pollen dispersal being the most effective. Successful interspecific hybridization is significantly enhanced using mixoploid *B. nigra* progenitor material. Gene flow rates in F1 hybrids were equivalent to those previously reported between *B. napus* with *B. rapa* and *B. juncea*, respectively, which are at the forefront of risk assessment concerns.

Keywords: *Brassica napus*, *Brassica nigra*, mixoploidy, gene flow, hybridization frequency, interspecific F1 hybrids

1. Introduction

Brassica napus (oilseed rape [OSR], rapeseed, and canola in a particular set of cultivars) is the most economically important of the *Brassica* crop species, and is currently the second most important oilseed crop worldwide [1]. OSR serves as the raw material for vegetable oil and extraction meal as feed and food, as well as mobility fuel for diesel cars and tractors, particularly in Europe [1]. Given the increasing international market demand for vegetable oil and biodiesel, transgenic OSR cultivation increased by 19% from 2016 to 2017 in the USA, Canada and Australia, to comprise 10.2 million ha of the estimated 36 million ha cultivated worldwide [2]. Transgenic OSR is reputed to be one of the potentially harmful crops in terms of gene flow due to a high outcrossing rate, high seed and pollen production, and long dispersal distances [3,4]. Concerns relating to the impact on agricultural and environmental ecosystems, as a result of potential gene flow from genetically modified (GM) OSR to widely-distributed, cross-compatible non-GM wild or weedy relatives, has received significant research focus [3-9]. Although *B. napus* is potentially able to hybridize with 23 wild relatives [5], research on the propensity for hybridization has been largely directed towards the

closest relatives, collectively represented by the well-known triangle of U [10], in which *B. napus* (AACC, 2n= 38), *B. juncea* (AABB, 2n= 36) and *B. carinata* (BBCC, 2n= 34) are allotetraploids derived from three diploid species, *B. nigra* (BB, 2n= 16), *B. rapa* (AA, 2n= 20), and *B. oleracea* (CC, 2n= 18).

The likelihood of intraspecific hybridization of GM *B. napus* with non-GM wild and domestic relatives of the same species has been substantiated as the most probable [4,8]. Interspecific hybridization of *B. napus* with recipient species, ranked in order of plausible possibility, were *B. rapa, B. juncea, B.oleracea, B carinata* and *B. nigra*, respectively [11]. This original ranking scheme was corroborated by subsequent research, particularly relating to the production of viable interspecific hybrids between *B. napus* and the recipients, *B. rapa* and *B. juncea*, respectively, and in some cases the apparent stabilization of genes over several generations by introgressive hybridization [3-9,12,13].

Earlier studies indicated either a very low probability or no success in producing viable hybrids between *B. napus* and *B. nigra* (black mustard), leading to the conclusion that interspecific hybridization and subsequent introgression between the latter under field conditions would be virtually non-existent [6,14,15]. Obstacles to successful interspecific hybridization between *B. napus* and *B. nigra* include the lack of a common chromosome set, in contrast to that of *B. rapa* and *B. juncea* which share the AA chromosome set with *B. napus*, as well as differences in ploidy level. A low hybridization frequency (less than 1 %) in the production of trigenomic hybrids was, however, reported between *B. napus* and *B. nigra* [16], as was the potential possibility of compatibility between *B. nigra* and hybrid populations derived from GM *B. napus* [8].

Overall, studies investigating the possibility of hybridization between GM *B. napus* and *B. nigra* have been scarce [8]. Of fundamental interest, was the presence of mixoploidy in wild *B. nigra* growing in the Ukraine [17]. The modal chromosome number was 32 and 24 in 50 % and 19 % of the plants, respectively, with remainder showing the well-documented diploid number of 16 [17]. This raises the interesting possibility of whether the propensity of interspecific hybridization between

B. napus and *B. nigra* may have been largely underestimated in the relatively few studies conducted thus far. Given that transgenic OSR field trials were already permitted in China [3], and authorization for the worldwide cultivation and commercialization of GM *B. napus* has now been granted [2], and that *B. nigra*, (particularly widespread in North America, Europe and parts of Asia) is described by the Invasive Species Compendium (ISC, www.cabi.org/isc) as a moderately invasive species (and in certain environments in the USA as a noxious weed), the presence of *B. nigra* as a recipient of gene flow cannot be overlooked.

To test the working premise that a significant interspecific hybridization frequency is possible, the objective of the present study was to investigate the hybridity of putative F1 seeds generated from multiple crosses between *B. napus* and a wild *B. nigra* population (collected in the Emilia-Romagna region, Italy) displaying mixoploidy. To this end, controlled hybridization between *Bt Cry1Ac B. napus* and 10 pollen recipient wild-growing *B. nigra* was conducted to test reproductive compatibility. The F1 progeny were assessed for viability, the presence and expression of the transgene, mixoploidy, as well as a range of seed and vegetative morphological features. Since it is not possible to perform open-field trials on GM plants, crosses between non-GM *B. napus* "Ceres" and wild *B. nigra* populations were made under open-field conditions. In these experiments, the effects of parental plant density and bee-mediated pollination were investigated.

2. Materials and Methods

2.1. Plant material

An OSR GM genotype cultivar "Westar" line GT 2-4 (*Bacillus thuringiensis (Bt) cry1Ac* endotoxin gene) [18] and a wild population of *B. nigra* (obtained from a local field collection in Emilia-Romagna) were the parental sources for the controlled hybridization experiments. The Westar *Bt* lines were developed by Halfhill *et al.* [19], to contain the 35S promoter of the Tobacco Mosaic Virus

(CaMV), a Green Fluorescent Protein marker gene (GFP), a kanamycin marker gene (nptII), the *(Bt) cry1Ac* endotoxin gene and the Nopaline Synthase Terminator (NOS-T) of *Agrobacterium tumefaciens*.

Given that that it is not possible to perform open-field experiments with GM plants, and OSR *B. rapus* cultivar "Ceres", provided by the Consorzio Agrario Bologna (Italy), and the same wild population of *B. nigra* were the parental sources for the spontaneous hybridization experiments conducted under field and, greenhouse conditions, respectively.

2.2. Brassica nigra (♀) x Bt Brassica napus (♂) *"Westar" line GT 2-4*

2.2.1. Growth chamber conditions and controlled hybridization

Numerous pollen donor *B. napus* and pollen receptor *B. nigra* plants were individually grown in plastic pots (20 cm x 12 cm), containing peat and fine sand (2:1 w/w). Pots were placed in a growth chamber (12 h photoperiod at 250 mmol photons m^{-2} s^{-1}, 20 °C day / 15 °C night). Throughout the growth cycle, plants were irrigated and fertilized with Hoagland solution containing: 5 mM KNO_3, 5 mM $Ca(NO_3)_2 \cdot H_2O$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and 0.02 mM $FeSO_4 \cdot 7H_2O$. A mix containing 0.02 mM Na-EDTA, 0.045 mM H_2BO_3, 0.01 mM $MnCl_2 \cdot H_2O$, 0.8 µM $ZnSO_4$, 0.3 µM $CuSO_4 \cdot 5H_2O$, and 0.1 µM $NaMoO_4 \cdot 2H_2O$ was also added. The phenological growth stages of *Brassica* were identified using the Biologische Bundesanstalt, Bundessortenamt, CHemical (BBCH) coding system [20]. At the stage of side shoot formation (BBCH code 22-23), plants were vernalized for 4 weeks (8 h photoperiod [at 250 mmol photons m^{-2} s^{-1}] at the constant temperature of 3 °C to stimulate simultaneous flowering. At the end of the vernalization period, growth conditions were set as follows: 14 h photoperiod (250 mmol photons m^{-2} s^{-1}), 20 °C day/ 15 °C night. At flowering (BBCH code 60-65), 10 *B. nigra* plants were emasculated and pollinated with a small brush by using the pollen collected from *Bt B. napus* stamens. At seed maturity (BBCH code 79), all the seeds produced by the 10 *B. nigra* parental plants were collected.

2.2.2. Germination and growth chamber conditions of F1 hybrid seedlings

All putative F1 hybrid seeds collected were gently rubbed in a double layer of sandpaper and placed in closed containers containing moist sand for cold stratification (6 weeks dark incubation at 3 °C). At the end of cold stratification period, the containers were placed in a growth chamber (12 h photoperiod, 18 °C). Viable (germinated) F1 seeds were individually grown in plastic pots, as described previously for the parental plants. The tetrazolium test [21] for viability estimation was conducted on all non-germinated seeds. At the growth stage of three detectable side shoots (BBCH code 23), a 200-mg leaf sample was collected from each F1 hybrid for determination of the *Cry1Ac* DNA amplicon.

2.2.3. Bt *transgene detection in F1 in hybrid seedlings*

The genomic DNA of the F1 hybrids was extracted according to the method proposed by Saghai-Maroof *et al*. [22]. The specific DNA primers used to amplify a 440 bp amplicon of the *Bt* transgene were Cry1Ac/Ab-F (5'-CACCACTATCTGTTCTTGACGGG-3´) and Cry1Ac/Ab-R (5'-ATACCGTACACGAACTCGATATC-3') [23]. Each amplification included 40 ng of template DNA, 1x PCR Buffer (Fermentas), 2.0 mM $MgCl_2$, 200 μM dNTP (dCTP, dGTP, dATP and dTTP), 0.5 μM for both primers and 1U of Taq DNA Polymerase (Promega), for a final reaction volume of 20 μL. The samples were amplified in a Whatman Biometra T gradient Thermal Cycler with the following temperature profile: initial denaturation at 95 °C for 12 min, followed by 40 amplification cycles at 95 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s. The reaction was concluded at 72 °C for 10 min. The PCR amplicons were analyzed on 1.8 % agarose TBE /ethidium bromide gels, along with a standard 100-bp (MBI Fermentas) ladder (100 to 3000bp). The specificity of the employed

primers was checked by sequencing the *Bt* 440 bp amplicon bands (Sequencing Service M-Medical, Milan).

2.2.4. Bt *transgene expression in F1 in hybrid seedlings*

Determination of *Bt* transgene functionality was determined by testing for the expression of the Cry1Ac protein in leaf tissues, using a double sandwich enzyme-linked immunosorbent assay (ELISA) QuantiPlate kit Cry1Ab/Cry1Ac (EnviroLogix Inc. Portland, Maine). Leaf pieces (50 mg) extracted from the F1 hybrids, positive for the 440 bp amplicon, were homogenized in 5 mL of the kit extraction buffer, centrifuged and the extract was diluted 1:50 (v/v) with the kit extraction buffer. All samples were centrifuged before addition to the ELISA plate. Spectrophotometric measurements were conducted with a plate reader (Labsystem Multiscan, Dasit, Italy) at 405 nm. *Bt*-toxin concentrations were expressed in ppb (µg Cry1Ac protein/kg fresh weight), as determined from a calibration curve based on standard *Bt*-toxin determination between 0.5 and 4 ppb.

2.2.5. Cytological analysis

The somatic chromosome numbers of 10 *B. nigra* plants, 10 *Bt B. napus* plants and all F1 interspecific hybrids, positive for the expression of the Cry1Ac protein, were determined from ovary tissue of young flower buds according to the procedure of Li *et al.* [24]. Chromosomes were counted in a total of five somatic cells under a microscope (ZEISS, Germany).

2.2.6. Seed and vegetative morphological features

Five replicates of 20 seeds were randomly selected from seed batches of the parental plants, *B.nigra* and *Bt B.napus*, respectively. Of the total number of putative F1 hybrid seeds, five replicates of 20

seeds were similarly analyzed prior to conducting the viability tests. For each replicate, seeds were weighed with a 5-decimal balance (Sartorius). Seeds were then placed on a silicone gel support and both sides of each seed scanned at 720 dpi using a flatbed scanner, according to Sako et al. [25]. The seed images were processed using the image analysis software Assess (American Phytopathological Society Press) in order to obtain both color intensity (expressed on a 0-255 arbitrary scale) and structural measurements. Structural characteristics included seed width (minor axis), height (major axis), area and perimeter, expressed as cm. From the ratios of various structural measurements, roundness and elongation were determined. The roundness (Ro) was calculated according to the following formula: $Ro = (4 \times \pi \times \text{area}) / \text{perimeter}^2$. If the roundness was equal to 1, the object was considered a perfect circle, with departure from a circular form occurring and as the ratio decreased from 1 [26]. Elongation was obtained dividing the seed height by the seed width. If the elongation was equal to 1, the object was considered roughly circular or square, with elongation increasing as the ratio decreased from 1 [26].

Vegetative morphological features of 20 randomly selected plants of both *B.nigra*, and *Bt B.napus*, respectively, as well as all F1 hybrids, positive for the expression of Cry1Ac protein, were evaluated by visual assessment at the growth stage of inflorescence emergence (BBCH code 50). Five evaluation descriptors, namely leaf color, leaf shape, leaf roughness, leaf and stem hairiness, were considered [27].

2.3. Brassica nigra (♀) x Brassica napus (♂) *variety "Ceres"*

2.3.1. Field experiment

A field trail was carried out to investigate spontaneous hybridization between non-GM *B. napus* "Ceres" and *B. nigra*, at the experimental farm of the University of Bologna, Cadriano (latitude 44° 33' N, longitude 11° 21' E, 32 m a.s.l.). Soil type was fine silty, mixed mesic, Udic Ustochrepts, with a silt loam texture, containing a ratio of 380, 375, and 245 g/kg of sand, silt, and clay, respectively.

Soil pH was 7.9. The experiment was a randomized complete block design with three replicates for each treatment combination. Each block (12 x 16 m) was composed of 9 experimental plots (2 x 4 m), respectively. The principle factor was sowing density ratio between *B. napus* and *B. nigra*, and was comprised of three ratios: 50 % : 50 % *B. napus* : *B. nigra*, 75 % : 25 % *B. napus* : *B. nigra*, 91.7 % : 8.3 % *B. napus* : *B. nigra*. For each sowing density, there were three experimental treatments: open-field, tunnel enclosure with solitary bees, and tunnel enclosure without bees.

In October 2014, the *Brassica* seed mixtures (respectively, diluted to obtain the principle factor ratios) were sown on each plot (surface area 8 m^2), comprising twelve rows (2 m in length, 0.30 m between rows, 0.02 m sowing depth), at a distance of 0.05 m within each row, for a total density of 60 seed m^{-2}. At the stage of stem extension, the plots were fertilized with $(NH_4)_2SO_4$ (100 g m^{-2}). Weeds were removed by hand, and neither herbicide or pesticide treatment was applied to plants in the field trial. In March 2015, prior to flowering each plot was manually thinned to obtain a final plant density of 50 plant m^{-2} (Supplemental Fig. 1). Non-woven polypropylene insect-proof nets (mesh size 0.2 mm) were used for the tunnel enclosure-treatments, and were erected immediately before the flowering stage (March 2015). A colony of solitary (Mason) bees (*Osmia cornuta* [Latreille 1805]) were provided by the Entomological area of the Department of Agricultural and Food Sciences, University of Bologna (Italy). A population number of 20 individuals was introduced into a single tunnel enclosure comprising the 3 solitary bee x plant ratio plots, respectively, within each block, during flowering to facilitate pollination. At seed maturity, only seeds from *B. nigra* in each plot were harvested manually. Seed samples were stored in a chamber at 10 °C and air humidity (50 % RH).

2.3.2. Greenhouse experiment

The trial was carried out in the greenhouses of the Department of Agricultural and Food Sciences, at the University of Bologna (Italy). Eight pollen donor *B. napus* cv. ‘Ceres’ and thirty-two pollen

receptor *B. nigra* plants were individually grown in plastic pots (30 cm x 15 cm), containing peat and fine sand (2:1 w/w). Pots were placed in a growth chamber (12 h photoperiod at 250 mmol photons m^{-2} s^{-1}, 20 °C day / 15 °C night). Throughout the growth cycle, plants were irrigated and fertilized with Hoagland solution containing: 5 mM KNO_3, 5 mM $Ca(NO_3)_2 \cdot H_2O$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and 0.02 mM $FeSO_4 \cdot 7H_2O$. A mix containing 0.02 mM Na-EDTA, 0.045 mM H_2BO_3, 0.01 mM $MnCl_2 \cdot H_2O$, 0.8 µM $ZnSO_4$, 0.3 µM $CuSO_4 \cdot 5H2O$, and 0.1 µM $NaMoO_4 \cdot 2H_2O$ was also added. Similarly, the phenological growth stages of *Brassica* were identified using the BBCH coding system [20]. At the stage of side shoot formation (BBCH code 22-23), plants were vernalized for 4 weeks (8 h photoperiod [at 250 mmol photons m^{-2} s^{-1}] at the constant temperature of 3 °C) to stimulate simultaneous flowering. At the end of the vernalization period, plants were placed in a ventilated greenhouse box (10 m x 8 m) and growth conditions were set as follows: 26 °C day / 18 °C night and natural sunlight. Two different experimental conditions were designed: one with free pollination and one with solitary bee-mediated pollination. For both pollination sets, pots were placed on the greenhouse bench according to the following scheme: one pollen donor *B. napus* pot was surrounded by 4 pots of *B. nigra* pollen, for a total of 4 pollen donor pots of *B. napus* and 16 pollen-receptor pots of *B. nigra* for each pollination condition (Supplemental Fig. 2).

As with the field experiment described previously, non-woven polypropylene insect-proof nets (mesh size 0.2 mm) were erected immediately before the flowering stage over the experimental plots designed to measure bee-mediated pollination, using solitary (Mason) bees (*Osmia cornuta* [Latreille 1805]). At flowering, a total number of 20 solitary bees were released into the enclosures to facilitate pollination. At seed maturity (BBCH code 79), only seeds from *B. nigra* in each experimental plot were collected.

2.3.3 Gene flow detection from non-GM OSR to black mustard in putative F1 hybrids

For the field trial, 600 viable *B. nigra* seeds for each experimental treatment combination (200 seeds per plot) amounting to 5400 plants, were grown in growth chambers as described previously for the *Bt* trial. For the greenhouse trial, a total of 1000 viable *B. nigra* seeds (250 per plot) amounting to 2000 plants, were similarly grown in a growth chamber. At the growth stage of three detectable side shoots (BBCH code 23), a 200-mg leaf sample was collected from each F1 hybrid for determination of gene flow, and genomic DNA extracted according to Saghai-Maroof et *al.* [22].

Prior to the determination of gene flow in the putative F1 hybrids, 30 Inter Simple Sequence Repeats (ISSR) primers, based on either non-anchored or anchored core repeats at either the 5' or 3' end, respectively, were screened for polymorphic loci in *B. napus* (cv. Ceres) and *B. nigra*. Three primers (LOL8 5'- GTGTGTGTGTGTCC-3', PHV2 5'–GACAGACAGACAGACA-3', BR6 5'–CACACACACACACACAGA- 3') were shown to produce 2 ISSR bands (amplicons of 650 and 590 bp, respectively), only detectable in *B. napus* genotypes, and were, therefore, used to determine the presence of gene flow from B. *napus* to B. *nigra* (Supplemental Fig. 3).

Each amplification reaction contained 20 ng template DNA, 100 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 4 µM primer (Genset SA, Paris, France) and 0.4 U of Taq DNA Polymerase (Promega) in a total volume of 25 µL. ISSR amplification consisted of an initial denaturation step at 94 °C for 1.5 min, followed by 35 cycles of 40 s at 94 °C, 45 s at 45 °C, 1.5 min at 72 °C, a cycle of 45 s at 94 °C, 45 s at 44 °C, and the final extension step of 5 min at 72 °C. Amplification products were separated and detected as described previously for the *Bt* experiment. A plant was considered a true hybrid if positive for all the two markers (amplicons of 650, and 590 bp).

2.4. Statistical Analysis

For the *B. nigra* (♀) x *Bt B. napus* (♂) controlled hybridization experiment, the seed and vegetative morphological characteristics between *B. nigra* x *Bt B. napus* and F1 hybrids was analyzed by one-

way ANOVA (Statistica 6·0 software 2001, StatSoft, Tulsa, OK, USA). Similarly, for or the *B. nigra* (♀) x *B. napus* (♂) spontaneous hybridization experiments, hybridization frequencies (%) of the number of viable F1 hybrids (containing *B. napus* gene material) for sowing density, as well as experimental treatment was analyzed by one-way ANOVA.

3. Results

3.1. Brassica nigra (♀) x Bt Brassica napus (♂)*"Westar" line GT 2-4*

3.1.1. Cry1Ac *gene flow from GM-OSR to black mustard under controlled conditions*

Cytological analysis of somatic ovary cells from 10 randomly selected *Bt B. napus* and *B. nigra* were analyzed for mixoploidy. The modal chromosome number of *B. napus* was 38, whereas mixoploidy was evident in the wild *B. nigra* population with a modal chromosome number of 32 and 16 for 70 % and 30 % of the samples, respectively (Table 1).

Ten plants from the mixoploid *B. nigra* population were randomly selected, emasculated, and subjected to a controlled cross with *Bt B. napus* as the pollen donor. All 10 *B. nigra* parental plants produced pods from crossed flowers, of which 82.6 % contained seeds (Table 2). A total number of 1371 putative F1 seeds were produced, of which 488 (35.6 %) produced viable putative F1 plants (Table 2). Both seed and viable F1 hybrid numbers varied significantly between the 10 *B. nigra* parental plants, of which three plants produced no viable hybrids. A total of 40 interspecific hybrids, derived from four *B. nigra* parental plants, were positive for the presence *Bt Cry1Ac* gene (Table 2), confirmed firstly by the presence of the PCR-generated 440 bp amplicon and followed by the sequencing analysis of the latter (Supplemental Fig. 4A). These viable F1 hybrids were considered true hybrids, as they contained the presence of the *Bt Cry1Ac* gene. Total hybridization frequency or hybridization rate (expressed as a percentage), as determined by the presence of the Bt Cry1Acgene

was found in 8.2 % (40 plants) of the viable F1 progeny (total number of viable 488 plants – Table 2). Of the 40 F1 hybrids containing the transgene, nine were positive for the expression of the Bt Cry1Ac protein (Table 2), as confirmed by Cry1Ab/Ac ELISA (Supplemental Fig. 4B). Hence, the hybridization frequency for the functionality of the transgene, amounted to 1.8 % of the total viable F1 hybrids. Interestingly, hybrids from parental plant numbers 5 and 8 were all positive for both the presence and the expression of the transgene. Cytological analysis of somatic ovary cells of the F1 hybrids positive for the expression of the *Bt* endotoxin showed a modal chromosome number of 35 for all 9 plants (Table 2).

3.1.2 Seed and vegetative morphological features

The morphological features of the putative F1 seeds were compared with the respective parental populations. Seed roundness and elongation factors were not significantly different between *Bt B.napus*, *B. nigra* and the F1 hybrids (Fig. 1). The F1 hybrids were significantly different from the *B. napus*, but not significantly different from *B. nigra* for seed colour, length width, perimeter, area and weight, (Fig. 1).

Vegetative morphology was compared between the parental material and the 9 true F1 hybrids positive for the expression of the *Bt* Cry1Ac protein. Fig. 2A shows the visual similarity between the F1 hybrids and *B. nigra* for leaf shape and rough adaxial leaf surface, as well as culm hairiness, in contrast to *B. napus*. For each descriptor evaluated, a decimal scale of arbitrary values was prepared. The maximum score (ten points) was assigned to the characteristics expressed by *B. nigra* (deep green leaf color, compound and elongated leaf shape, very rough adaxial leaf surface, very hairy adaxial and abaxial leaf surfaces, very hairy stem), whilst the minimum score (zero) was attributed to the characteristics expressed by *B. napus* (blue-green leaf color, rounded leaf shape, smooth adaxial leaf surface, scant hairiness of adaxial and abaxial leaf surfaces, hairless stem). Using the scale, it was possible to provide arbitrary scores to the F1 hybrids on the basis of vegetative similarity to the

parental plants. For all vegetative traits related to leaf colour, shape, texture, presence or absence of stem and culm hair, the 9 F1 hybrids expressing the *Bt* protein were all more similar to *B. nigra* than *Bt B. napus* (Fig. 2B).

3.2. Brassica nigra (♀) x Brassica napus (♂) *variety "Ceres"*

Gene flow from non GM OSR to black mustard in a field and greenhouse experiment

Field and greenhouse experiments were conducted with non-GM *B. napus* and hybridization was considered positive if true F1 interspecific hybrids from *B. nigra* seed were positive for the presence of two amplicons (Supplemental Fig.3.) derived from the *B. napus* genome. The plant density at flowering was set at 50 plants m^{-2} in each experimental plot in the field experiment, with ratios between *B. napus*: *B. nigra* ranging from 1:1, 3:1 and 11:1, respectively (Fig. S2). Interspecific hybridization frequencies (rates) varied significantly for plant ratio (Table 3), with the increased rates positively associated with the increasing presence of *B. nigra* under all experimental conditions. Hybridization frequencies in the open-field plots, simulating both wind-mediated and wild insect-mediated pollination were significantly higher than those obtained from treatments under insect-proof netting (Table 3). Since the non-woven insect-proof net material causes a reduction in air-flow [28], the enclosures simulated conditions with reduced wind-flow (compared to the open-field). No significant differences in hybridization frequencies were obtained between two enclosure treatments with or without the presence of solitary bees, respectively (Table 3).

Under greenhouse conditions, the solitary bee-mediated pollination resulted in increased hybridization frequencies compared to the non-insect treatment (Table 4). Overall hybridization frequencies in the field experiments (Table 3) were higher than those obtained in the greenhouse for the ratio of parental plants during the crosses (Table 4).

4. Discussion

The present study demonstrates, for the first time, a significant propensity of gene transfer from *B. napus* (AACC, ♂) to *B. nigra* (BB, ♀) in F1 hybrids under both controlled and spontaneous hybridization conditions. An elevated level of mixoploidy (70 % with a tetraploid modal chromosome number of 32) in the wild-growing population of *B. nigra* (collected in the province of Emilia-Romagna) is suggested to be the contributory factor. Of the *B. nigra* plants collected in the Ukraine, the majority were mixoploid, containing either triploid (n = 24) or tetraploid (n= 32) cells [17]. This raised the question of whether the presence of mixoploidy, inducing an elevated capacity for hybridization, would potentially increase the possibility for hybridization with *B. napus* [17]. The interesting possibility raised by those authors was confirmed by the present work, using a *Bt (Cry1Ac* gene*)* from *B. napus* (n= 38) and wild-type *B. nigra* (potentially n= 32), to produce F1 hybrids that were positive for the expression of the Cry1Ac protein. Total hybridization frequency, as determined by either by the presence of the *Bt Cry1Ac* gene or the more stringent measure of functionality of the expressed protein was either 8.2 % or 1.8 % of the F1 progeny, respectively. The hybridization frequency or rate was shown to be significantly higher than that reported for the successful controlled (manual pollination) hybridization between OSR and *B. nigra* in previous work published to date [5,16,29]. Similarly, spontaneous hybridization (field and glasshouse) experiments showed significantly higher hybridization frequencies or rates, that varied from *ca* 4 to 29 % (for the presence of gene material derived from *B. napus*) in F1 hybrids *of B. nigra* depending on the experimental conditions.

The higher hybridization frequencies in the present study were all obtained with *B. nigra* as the maternal parent, in contrast with previous research that showed some, albeit minimal, success being obtained when using *B. napus* and *B. nigra* as the maternal and paternal parent, respectively [5,16,29]. Many *Brassica* interspecies crosses are reputed to be more successful when the female parent has a higher ploidy level [16,30]. Mixoploid female parents, evident in the present study, would

meet the requisite of a higher ploidy level, thereby raising the possibility for successful interspecific hybridization. Previous studies, investigating the possibility of interspecific hybridization between OSR and black mustard, utilized registered varieties of *B. nigra* and not wild-growing populations [14-16]. Whether registered or commercial varieties of *B. nigra* display a higher chromosomal uniformity or stability, and are typically diploid (n= 16), necessitates further research but would nevertheless explain both the lack of success and the lower hybridization frequencies obtained from *B. napus* and *B. nigra* interspecific crosses reported previously in the literature. True F1 trigenomic hybrids (n= 27) were produced with a hybridization frequency of 0.27 %, only from crosses where *B. napus* and *B. nigra* accessions were exclusively used as the female and male parents, respectively [16]. Chromosome testing of *B. nigra* confirmed the diploid state (n= 16), thereby showing that the trigenomic hyrids received a haploid complement of 19 and 8 chromosomes from *B. napus* and *B. nigra* respectively [31]. Similarly, the true F1 hybrids (n= 35) in the present study would have received a haploid complement of 19 from *B. napus* and a haploid complement of 16 from a tetraploid (mixaploid) *B. nigra*.

Vegetative morphology of the F1 hybrids in the present study were more analogous to *B. nigra*, in color, shape, texture of the leaves, as well as the presence of hair on both abaxial and adaxial leave surfaces and the stem. In a single study, vegetative morphology for the trigenomic hybrids between *B. napus* x *B. nigra* was described as intermediate, although hybrids were positive for the presence of leaf and stem hairiness, traits that were absent in *B. napus* [16]. Noteworthy, all potential putative F2 trigenomic hexaploids and anueploids, derived from either self-pollination of the triploid hybrids or from the treatment of the latter with colchicine to induce polyploidization, were positive for leaf and stem hairiness, traits representative of *B. nigra* [31]. Given the scarcity of research on interspecific hybridization between OSR and black mustard [7,8], literature relating to morphology is lacking.

Hybridization frequencies of F1 hybrids in the field studies were shown to range from *ca* 4 to 30 %, depending on the experimental treatments, showing for the first time the production of F1

hybrids from *B. nigra* containing genetic material from *B. napus* following spontaneous hybridization between *B. nigra* and *B. napus* [8,14,15]. The hybridization frequencies of F1 hybrids obtained in the present study were similar for those obtained under field conditions for crosses between *B. napus* and *B. rapa* (*ca* 0.7- 37 %) and B. *napus* and *B. juncea* (*ca* 0.3 – 22 %), respectively [8,13,14,32-34], which constitute the two representative *Brassica* varieties ranked with highest propensity for interspecific hybridization with OSR [11]. The common set of chromosomes shared between *B. napus* and *B. rapa* enhance the likelihood of interspecific hybridization and gene flow, despite the differences in ploidy level. The present research suggests that barriers to interspecific hybridization resulting from the lack of common set of chromosomes, as is evident between *B. napus* and *B. nigra*, may be overcome by a raised ploidy level, thereby attaining hybridization frequencies within the ranges of that reported *B. rapa*. Similar to the results presented, natural interspecific hybridization between *B. napus* and either *B. rapa* or *B. juncea* varied widely, depending on the environment under which the plants develop and the design of the experiment.

A plant density of approximately 50 plants m^{-2} was used in the present field trial, within the recommendable range for *B. napus* for improving yield [35]. Sympatric ratios between the parental plants impacted significantly on the success of interspecific hybridization. The highest ratio (1:1 *B. napus* : *B nigra*) significantly increased degree of hybridization under all treatments, in turn steadily and significantly decreasing with the increase in the ratio of *B. napus* to *B. nigra* (3:1 and 11:1), corroborating previous work [33] for increasing ratios of B. *napus* to *B. juncea* (1:1 to 1:15). The majority of studies investigating the possibility of hybridization are designed on the basis of distances between the parental material [9]. Increased hybridization frequencies were evident when *B. rapa* was positioned within the center of the *B. napus* fields as a opposed to the margins [6,32,34].

The present study raises the interesting question of whether gene flow from *B. napus* into wild-growing *B. nigra* populations has been underestimated. The unanticipated speed of area wide expansion of some herbicide resistant (HR) weed biotypes has impelled numerous appeals over the past decade for a collective community or regional response to mitigate the unhindered spread of HR

alleles [36]. In the existing framework of gene flow into weedy relatives being underestimated [36], this aspect is warranting of attention. Risk assessment of the propensity of for hybridization between OSR and wild populations of weedy relatives in any environment of interest would necessitate obtaining information regarding the co-occurrence of weedy relatives in OSR fields, and degree of abundance (low, medium or high) both within fields and on field margins [6]. The possibility of interspecific hybridization both under controlled and field conditions between *B. napus* and *B. nigra* needs to be re-evaluated in risk assessment studies, taking into consideration the possibility of mixoploidy in wild-growing populations of *B. nigra*. Of great importance in future research will also be to evaluate the propensity of interspecific introgression of genetic material into the F2 and subsequent generations of hybrids.

The success of spontaneous interspecific hybridization, or gene flow from *B. napus* to *B. nigra* was significantly influenced by environmental conditions. Hybridization frequency under open greenhouse conditions was *ca* 3.5 % opposed to 29 % (at the highest ratio between parental material) of the open-field, reflecting the combined importance of wind- and wild-insect-mediated pollen dispersal, as has been reported previously [37,38]. The pollen of *B. napus* has been reported to be covered with pollenkitt, a viscous lipidic substance resulting in pollen clumps, which insect pollinators render airborne thereby contributing to "insect-assisted wind pollination" [37]. Reduced airflow in the plots covered with non-woven insect-proof net, despite the presence of solitary bees within, attest to the importance of wind-mediated pollination in promoting successful interspecific hybridization. However, the relative efficacy of wild-insect pollinators compared to solitary bees under the experimental conditions was not tested. Solitary bees, previously shown to be more efficient than honeybees in pollen transfer per unit visit [39], were only marginally more effective in promoting successful interspecific hybridization that the experimental control of reduced wind and no solitary bee-mediated pollination, once again suggesting the importance of "insect-assisted wind pollination". The present study contrasts with that of Zhang et al. [13], indicating that the contribution of honeybees exceeded the contribution of wind to the successful hybridization between *B. napus* and *B. juncea*

over short distances. However, according to an extensive review of literature published between 1956 and 2018 on insect-mediated pollination of *B. napus*, results (conducted primarily on honeybees) showed the efficacy of pollination varied significantly according to geographical regions, field conditions and plot size, making effective comparisons on the contribution of bee-mediated pollination difficult [40].

5. Conclusions

The present study has shown that the propensity of successful interspecific hybridization between *B. napus* and *B. nigra* is significantly enhanced in mixoploid *B. nigra* progenitor material. The elevated ploidy level in mixoploid female *B. nigra* parents is suggested to raise the possibility for successful interspecific hybridization. Interspecific hybridization rates in F1 progeny, using a *B. nigra* progenitor population with a modal chromosome number of 32 in 70% of the population, were equivalent to the those previously reported between *B. napus* and *B. rapa* and *B. juncea*, respectively (under both controlled and open-field conditions), which have been a central focus in risk assessment studies on *Brassica*.

Declaration of competing interest

The authors declare not conflict of interest.

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Appendix A. Supplementary data

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Table 1 Chromosome number (for a total number of 5 somatic cells) in 10 plants of *Brassica napus* and *Brassica nigra*.

	Brassica napus		*Brassica nigra*	
Plant No.	Chromosome no. determined	Modal number	Chromosome no. determined	Modal number
1	38 (5)	38	16 (5)	16
2	38 (5)	38	16 (5)	16
3	38 (5)	38	16 (5)	16
4	38 (5)	38	32 (5)	32
5	38 (4) , 37 (1)	38	32 (5)	32
6	38 (5)	38	32 (5)	32
7	38 (5)	38	32 (5)	32
8	38 (5)	38	32 (5)	32
9	38 (5)	38	32 (4), 24 (1)	32
10	38 (5)	38	32 (4), 24 (1)	32

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Table 2 The total number of flowers crossed, pod and seed set, viable F1 hybrids, and F1 hybrids that were positive for the presence and expression of the *Bt* transgene and with a modal chromosome number of 35 from 10 maternal *Brassica nigra* plants crossed with *Bt Brassica napus*.

B. nigra Plants	Flowers crossed	Pod set	Pods with seed	Putative F1 seed	F1 seed /pollination	Viable F1 hybrids	Cry1Ac gene present in F1	Cry1Ac toxin expression in F1	F1 Chromosome modal no. n =35
1	48	32	19	85	1.0	0	0	0	
2	56	50	49	279	5.0	190	31	1	1
3	43	30	27	20	0.5	0	0	0	
4	33	22	16	15	0.5	0	0	0	
5	48	43	38	259	5.4	174	4	4	4
6	44	38	23	202	4.6	8	0	0	
7	51	39	27	82	1.6	14	0	0	
8	44	40	36	22	0.5	19	4	4	4
9	58	54	50	261	4.5	19	1	0	
10	44	38	34	146	3.3	64	0	0	
Total	469	386	319	1371		488	40	9	9

Table 3 Hybridization frequency and number of F1 hybrids positive for *Brassica napus* genetic material from a *Brassica nigra* (♀) x *Brassica napus* (♂) cross at different ratios of parental material exposed to open-field-mediated pollination, reduced wind and solitary bee-mediated pollination and the control (reduced wind and no bee).

		50 : 50 *B. napus : B. nigra* *a		75 : 25 *B. napus : B. nigra* *b		91.7 : 8.3 *B. napus : B. nigra* *c	
Experimental treatment	F1 hybrids (replicates)	*B.napus* DNA present (No.)	Hybridization Rate (%)	*B.napus* DNA present (No.)	Hybridization Rate (%)	*B.napus* DNA present (No.)	Hybridization Rate (%)
Open-field **a	200	60	30.0	22	11.0	6	3.0
	200	71	35.5	18	9.0	7	3.5
	200	45	22.5	16	8.0	11	5.5
Total No. Mean (%)	600	176	29.3	56	9.3	24	4.0
Net enclosure: Bee **b	200	34	17.0	10	7.0	1	0.5
	200	29	14.5	14	5.0	0	0
	200	25	12.5	16	8.0	1	0.5
Total No. Mean (%)	600	88	14.7	40	6.7	2	0.33
Net enclosure: control **b	200	24	12.0	12	6.0	0	0
	200	16	8.0	8	4.0	0	0
	200	24	12.0	4	2.0	0	0
Total No. Mean (%)	600	64	10.7	24	4.0	0	0

**$P < 0.001$ –(open field –enclosed honeybee, enclosed control), *$P < 0.05$ (sowing density)

Table 4

Hybridization frequency and number of F1 hybrids positive for *B. napus* genetic material from a *B. nigra* (♀) x *B. napus* (♂) crosses exposed to the presence and absence of solitary bee- mediated pollination in a greenhouse.

Experimental treatment	F1 hybrids (replicates)	20 : 80 *B. napus : B. nigra* *B. napus* DNA present (No. of plants)	Hybridization rate (%)
Open * b	250	11	4.4
	250	10	4.0
	250	10	4.0
	250	5	2.0
Total	1000	36	3.6
Enclosed: Bee *a	250	17	6.8
	250	20	8.0
	250	11	4.4
	250	21	8.4
Total	1000	69	6.9

*$P < 0.05$ (experimental treatment)

Fig. 1. Comparison of seed morphological characteristics between *Brassica napus*, *Brassica nigra* and interspecific F1 hybrids.

Fig. 2. Comparison of vegetative morphological characteristics at the 8-10 true-leaf stage between *Brassica napus*, *Brassica nigra* and interspecific F1 hybrids. (A) photographic comparisons for

752 leaf shape, texture and hairiness. (B) Visual scoring of morphological descriptors of the F1

753 hybrids relative to similarity to either *B. napus* (0 units) or *B. nigra* (10 units).

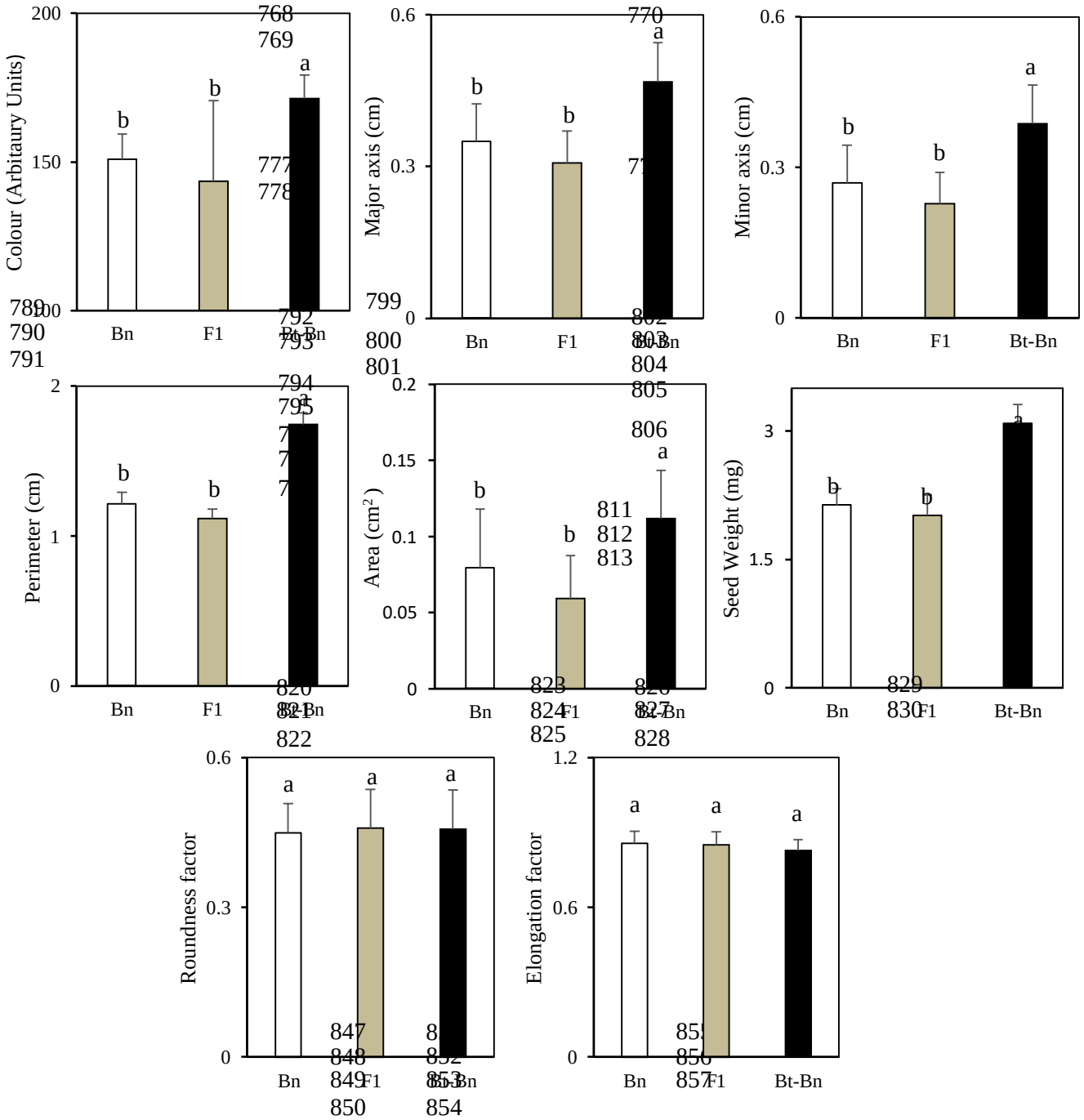
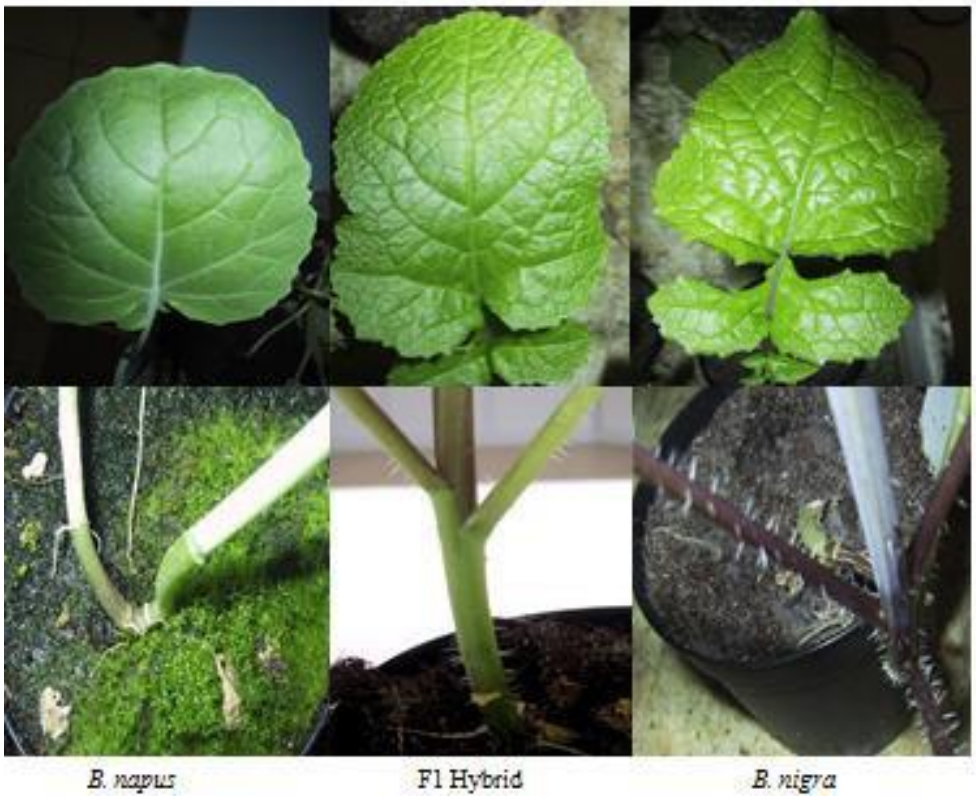

Colour (Arbitaury Units)
200
150
100
b
b
a
Bn
F1
Bt-Bn
Major axis (cm)
0.6
0.3
0
b
b
a
Minor axis (cm)
0.6
0.3
0
b
b
a
Perimeter (cm)
2
1
0
b
b
a
Area (cm²)
0.2
0.15
0.1
0.05
0
b
b
a
Seed Weight (mg)
3
1.5
0
b
b
a
Roundness factor
0.6
0.3
0
a
a
a
Elongation factor
1.2
0.6
0
a
a
a

Fig. 1.

A



B

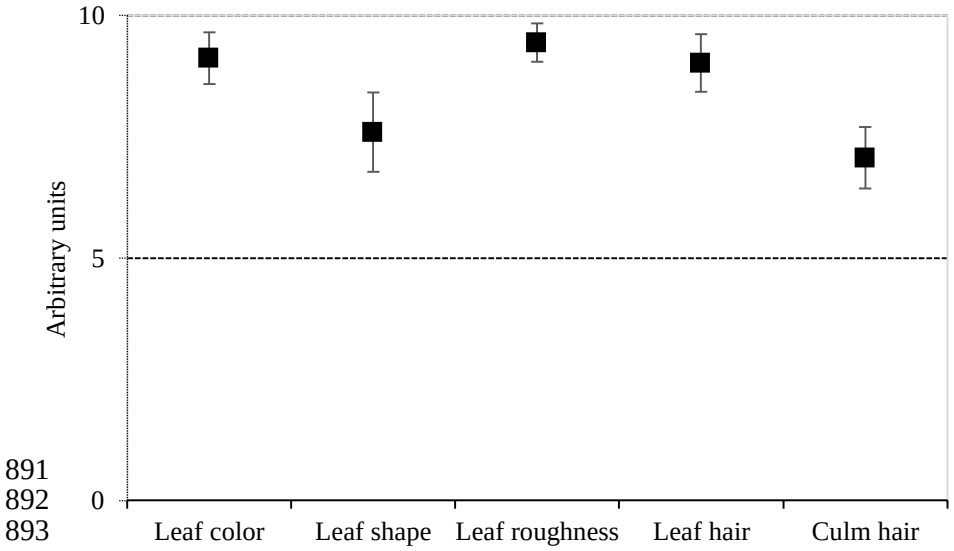

10
5
0
Arbitrary units
Leaf color
Leaf shape
Leaf roughness
Leaf hair
Culm hair

Fig. 2.