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Molecular Fingerprinting Confirms Pollen-Proofing of Nonwoven Pollination Control Fabrics in Sugar Beet

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ABSTRACT

We tested the pollen-proofing ability of three new pollination control tents (PCTs) made from nonwoven fabrics (DWB10, DWB23, DWB24) developed to have an open architecture to improve light and air permeability while still maintaining an effective barrier to pollen compared to standard duraweb® (DWB01) fabric. During 2020 and 2021 two methods of evaluation were used at Lion Seeds Ltd, Essex, UK on single potted plants of a cytoplasmic-genetic male sterile family (CMS): (a) fingerprinting of parent and progeny genotypes of seed set from CMS plants under PCTs, using 209 molecular markers, (b) analysis of seed-related traits. Adjacent open-pollinated plants showed high seed set indicating abundance of ambient pollen, while those under PCTs saw 86% less seed weight and 96% less implied seed numbers (ISG) showing that 'non-seeds' were collected as seeds. Molecular markers analysis of two years of PCT progeny showed: 1. non-significant difference between PCTs for percent homozygosity; 2. Parent vs. progeny percent homozygosity was significant in 2020 (85% of parent vs. 77% of progeny) but not in 2021 (73% in parent and progeny); 3. The CMS family was not pure-breeding and the mean homozygosity of 75% over two years was a good fit for theoretical expectations of one generation of inbreeding. Analysis (b) of various seed-related traits showed non-significant differences between PCTs except for 1000-seed weight and germination percent. The implied seed number weighted by germination percent was virtually zero for all PCTs. Both (a) and (b) confirmed that novel PCT fabrics despite greater air permeability, were as pollen-proof as the control DWB01

providing new options in sugar beet breeding to maintain plant health. Highlights: Analysis of molecular markers and seed related traits confirmed pollen proofing of new nonwoven fabrics with more open architecture and greater strength for pollination control in sugar beet.

Keywords: Molecular markers, male sterility, sugar beet, nonwoven fabric, pollination control tent.

INTRODUCTION

Townson et al. [31] compared the performance of cytoplasmic-genetic male sterile (CMS) plants grown in open and mini-isolation tents and tested the pollen proofing and effects of micro-climate within nonwoven pollination control tents on agronomic traits. They tested the hypotheses: (a) the mean number of seed set on CMS plants grown in tents to return a near-zero estimate within the statistical error limits, provided the tents were pollen-proof and did not allow foreign pollen intrusion. (b) morphological plant traits of the CMS plants in tents performed like those in the open control (H0), provided the micro-environment within tents did not influence plant traits differently than in the open control. In both cases, they reported the acceptance of the null hypothesis (H0).

The present study extended the 2019 experiments of Townson et al. [31] for another two years during 2020 and 2021 to collect further evidence for pollen proofing ability of four tent fabrics based on molecular markers and the rate of seed set. Consistently, in all years, a commercially exploited male sterile (CMS) family was used as it produces non-functional pollen [6]. Its male sterility is cytoplasmic-genetic type [18] in which MS (male sterile) or A-line (family) is maintained with an O-type maintainer family or B-line, and a pollinator with restorer gene (R-line) is used for hybrid seed production. Under circumstances of perfect pollen exclusion and complete sterility no progeny would be expected, provided there exists no environmental sensitivity of the male sterility gene [22]. The frequency of viable seeds would then indicate the ability of the barrier materials to exclude pollen. However, as in other crops, the stability of cytoplasmic male sterility expression in sugar beet (*Beta vulgaris* L.) is reported to be environmentally sensitive in specific genotypes [17]. Pollen sterility may break-down under certain environmental conditions or due to the unintended presence of fertility restoring factors in the nuclear genome (rare off-types usually due to contamination during production of the CMS family). A comparison of the molecular marker fingerprint between each CMS and associated progeny would confirm if they were the product of a selfing event (CMS failure) or a cross (pollen exclusion failure).

Clifton-Brown et al. [7] reported the average size of sugar beet pollen to be ~20-25 µm but according to Hecker [13] the mean diameter of pollen of diploid (2x) strains of sugar beet was 20.8 µm (19.3 to 22.5 µm) and that of auto-tetraploid (4x) to be 25.9 µm (23.4 to 27.4 µm). Both wind and insects assist in sugar beet cross-pollination [3] and the wind may carry the pollen as long as 1200 m [8]. Usually, pollination bag materials with porosity smaller than the pollen size are used to avoid contamination [12]. More open architecture increases airflow and light penetration, helping to improve legitimate pollen dispersal, plant vigour and reduce humidity inside the tents and improving disease resistance and seed set. Stronger fabrics are also

required for larger pollination control tents. In this study, nonwoven fabrics of at least three new tent types were deliberately chosen for improved strength and more open architecture than the standard being used at Lion Seeds. This was envisaged to allow a trade-off between pollen-proofing and breathability for a more ambient micro-climate adjustment within tents. The major objectives of the present study were to: (i). Compare and identify new nonwoven pollination control tent fabrics with pollen-proofing ability and (ii). Assess if the nonwoven fabrics with more open architecture retain pollen-proofing ability suitable for use in sugar beet hybrid breeding. The study uniquely employed molecular markers and seed-related traits to test the pollen-proofing ability of synthetic fabrics.

MATERIALS AND METHODS

Genetic Material

Lion Seeds provided MS plants from families bred for commercial use. Sugar beet breeding families are not developed to be inbred lines but to be reasonably phenotypically uniform and true-breeding, and near-homozygous for practical purposes. Such families may depict within-family variation that is a joint effect of non-genetic or environmental and residual genetic variation.

Experimental Details

Details of five treatments (mini tents) remain as given in Townson et al. [31]. Briefly, these included three new nonwoven synthetic fabric treatments (DWB10, DWB23, and DWB24), standard DWB01, and open control without any cover. The standard DWB01 has been used at the Lion Seeds for many years. It is heat-bonded fabric which retains a flexible nature that makes it easier to handle. The three new fabrics are spun bond and have greater strength and air permeability combined with an architecture that impedes pollen penetration.

Figure 1: Field arrangement of tent treatments and controls (left) and a close up of a mini-tent covering a single sugar beet plant of male sterile line (right). A window on the side of mini-tent facilitated the view of the plant in the cover.

The new fabrics have a larger pore size then DWB01 but are stronger making them more suitable for larger isolation structures. Increased air permeability maintains an internal environment more consistent with external, with greater light transmission at the usable spectrum range of 350 to 800 nm. These features are intended to optimize internal conditions for better seed set and the architecture of the fabric provides the necessary barrier against external pollen.

Each mini tent measured 63.5 x 63.5 cm and 120 cm high with a 20 cm skirt at the base. Each tent covered a single plant (Figure 1). The plants were grown in a 22.1 m long row with 50 cm spacing between them. Each treatment was allocated five potted plants that were completely randomised among all treatments. The tent covers were placed on iron frames which were anchored in the ground and the covers were adequately fixed with sandbags on skirts to ensure no pollen entry underneath the skirts [31].

The experimental plants were surrounded by flowering sugar beet plants in adjacent poly-tunnels to generate sufficient pressure of wind-born pollen. The experiment was conducted over three years during the 2019, 2020, and 2021 summer seasons at Lion Seeds in Essex, UK. CMS plants were checked for sterility by examining the first developing flowers and for a consistent family phenotype before transfer to isolation. The plants were enclosed in tents during the first fortnight of June and harvested in August each year. During 2019 plants were enclosed on the 10th of June and harvested on the 9th of August. During 2020, plants were caged on 24th May and harvested on the 4th of August. The plants were caged on 16th June and harvested on 18th August during 2021.

Molecular Markers

Seedling Growth and DNA Extraction for Marker Genotyping:

Seeds collected from plants under each tent type were the progeny seeds and were germinated. The seedlings so produced were transferred to trays of potting compost. Leaf samples for DNA extraction were collected from the first true leaves and transferred to a collaborating laboratory at the University of Padova for DNA extraction and marker genotyping. Leaf tissue of the CMS parent plants had been sent for the same purpose prior to the isolation of plants with the tent treatments.

Mining SNPs:

The strategy for the selection of SNPs utilised RAD-Sequencing data of Lion Seeds CMS and Pollinator lines. These were supplemented with publicly available and reported SNPs from https://bvseq.boku.ac.at/PhysMapC/markers.fasta.txt. A total of 210 SNPs were short-listed.

SNP Assay Design:

Flanking sequences of 200 bp on either side of the selected SNPs were used to evaluate their suitability for AgriSeq customized panel design and quality control process. The quality check was performed using the EL10.1 reference genome available at NCBI (accession: GCA_002174835.2) and then submitted to the primer design phase. The primer designs were in-silico checked for specificity and sensitivity of the intended target/marker regions

using the sugar beet reference. Finally, 210 SNPs passed the design thresholds to constitute a customized sugar beet panel and 209 were used.

DNA Extraction

Automated genomic DNA isolation was carried out using the BioSprint 96 workstation (Qiagen, Hilden, Germany) according to the method described by Stevanato et al. [23, 29, 30].

Library Preparation, Templating and Sequencing

Samples were prepared for sequencing using the AgriSeq HTS Library Kit (Thermo Fisher Scientific). In short, DNA concentrations were normalized to 3.3 ng/μL for a total of 10 ng DNA per 10 μL reaction. Normalized DNA was combined with the AgriSeq custom primer panel and AgriSeq amplification master mix. For amplification of genomic targets, the following thermocycling programs were used; 99°C for 2 minutes, then 15 cycles of 99°C for 15s and 60°C for 4 minutes. Amplicons were prepared for ligation with pre-ligation enzyme digestion at 50°C for 10 minutes, 55°C for 10 minutes, and 60°C for 20 minutes. IonCode™ Barcode Adapters 1-384 Kit (Thermo Fisher Scientific) were ligated to the digested products with barcoding enzyme and buffer. Labeled amplicons were then pooled, cleaned up, amplified, and normalized. Following library preparation, libraries were enriched on Ion sphere particles using an Ion 540™ Chip Kit on the Ion OneTouch™ 2 System. Sequencing was performed on the Ion S5 system (Thermo Fisher, Inc. Waltham, MA). After sequencing, genotyping was performed automatically by Torrent Variant Caller (TVC) on the Torrent Suite Server (TS).

Genotyping

The variant calling pipeline of Ion Torrent sequencing data was performed on the Torrent Suite Server (Thermo Fisher Scientific). First, the signal processing files were automatically transferred from the sequencing platform to the S5 server and then converted to raw reads (FASTQ). Following this, the sequenced reads were de-multiplexed to individual samples using the barcode sequences. For each sample, the sequenced reads from the targeted regions were mapped to the sugar beet EL10.1 reference genome using TMAP- Torrent Mapping Alignment Program followed by genotyping using TVC-Torrent Variant Caller. The tool utilises a bed format file of intended SNPs and HOTSPOT regions to provide genotyping calls. The genotypes of all samples are finally reported in TOP/BOT format were used for analysis of the genotypes downstream.

In 2021 a total of 20 CMS plants were used to test 5 replications of 4 cover treatments. 18 of those plants returned a total of 1229 progeny that needed to be qualified as the product of selfing or crossing. For genotyping, 209 single nucleotide polymorphism (SNP) markers were selected for reliable performance and broad distribution across the genome.

The genotypes of the subset of markers that were homozygous in the parental CMS plants were compared with those of any germinated seedlings in a sample after harvest. Markers homozygous in the parent were selected because they would be diagnostic in the sense that a different genotype in the progeny would discount selfing as the causative pollination event. Given a large enough number of such markers, an identical genotype in a progeny would be more likely the product of a self-pollination event than an out-cross.

For each recovered seedling, the genotype of each diagnostic marker was compared with the parent CMS and the result classified as:

- P – marker genotype same as parental
- X – marker genotype not possible from parental self but possible from a cross (genotype of progeny different to CMS). This genotype cannot be due to selfing and depending on the degree to which we can discount seed contamination or call-error, represents pollen introgression.
- C – marker genotype not possible from self or cross. This genotype can only be possible due to seed contamination or 'call error' in the parent or progeny genotype reaction.
- 0 – marker failed to return genotype in CMS or progeny.

Comparison of the number of markers returning the P, X and C classifications were used to judge the likelihood of the progeny being produced by self-pollination or out-crossing.
The number of markers that failed were excluded from the total of 209 markers to give the number of True Total Diagnostic (TTD) markers. The Total Diagnostic homozygous (TD, 11+22) makers in parents were expressed as percent homozygous makers of the TTD. The parental (P) type markers in the progeny were expressed as percent of the number of TD markers in the parent.

The percent homozygous markers in parents and progeny of seed set in the covers were subjected to analysis of variance, using Minitab 21 software, to test if the progeny homozygosity level differed from the parental plants.

Seed Related Morphological Traits

Harvested material from each plant was divided into below 2.8mm and above 2.8mm with a circular hole sieve. The latter were taken as prospective seeds and weighed. These were divided in four replications of 100 seeds and weighed separately and 1000-seed weight was derived. Each of these four replications of 100 seeds per plant was sown for the germination test. Germination was recorded on 2^{nd}, 3^{rd}, 7^{th}, and 10th day after sowing and overall germination over four replications in percent was computed after 10 days. The germination certainly is an important aspect of the real seed. What is recorded as seed weight is just the mass of 'seed sized material' that was recovered by the sieving procedure. In many cases, aborted flowers will dry down into small seed shapes that are recovered but fail to germinate as they are not real and viable seeds [31]. Sugar beet breeders usually expect at least 10g of seed in an open pollination with 75% germination during a typical year.

Derived implied seed number was computed as below:

Implied Number of Seeds (IS):

Any seed-like material with >2.8mm (diameter) was taken as probable seed and weighed together for each plant in grams (X).

Four samples of 100 seeds were taken and weighed, and weight for 1000 seeds was extrapolated from weight of four hundred seeds in grams (Y).

Implied number of seeds (IS) was computed using X and Y

$$\text{IS} = \left(\frac{X}{Y}\right) * 1000$$

Implied Total Number of Germinated Seeds (ISG):

Number of seeds obtained from materials that looked like seed may be misleading. If it were a viable seed then it should germinate. Therefore, the number of implied seeds that could germinate by 10 days were computed to find out the actual number of seeds per plant as:

$$\text{ISG} = IS * \left(\frac{Germ\,\%}{100}\right)$$

A combined analysis of variance was performed over two years (2020 and 2021). The SS for 4 df between cover types was partitioned into two orthogonal components: 1. Control vs covers for 1 df and 2. Between covers for 3 df. The first item (1) was computed using orthogonal polynomials while second item (2) was computed by subtraction from the Sum of Squares (SS) for cover treatments. It was observed that most of the differences between the mean values of the four fabrics of covers were discernible into two groups of: (DWB01 and DWB10) as Grp 1, and (DWB 23 and DWB24) as Grp 2. Therefore the 'Between covers' SS for 3 df was further partitioned into Grp1 vs Grp 2 for 1 df and the remainder for 2 df was computed by subtraction (Table 1).

RESULTS

Molecular Markers

Analysis of variance for homozygosity (%) in parent plants and their progeny revealed that Identity or Parents vs. Progeny item was significant in 2020 only which could be the result of some tents showing manufacturing defects and possibility of pollen intrusion. However, tent types or their interaction with identity were consistently non-significant over two years (Table 1).

Table 1: Mean squares (Ms) from year-wise analysis of variance for homozygous (%) markers of the 209 markers tested in parent and progeny under four fabrics of tents. Total diagnostic markers of 11 + 22 types for a biallelic situation were used for computing homozygosity percent in the parent. Homozygous of these in the progeny was used to compute the percentage of homozygosity in the progeny.

Source	df	MS 2020	P	df	MS2021	P
Identity (Parent vs progeny)	1	714.81	0.00	1	0.13	0.95
Tent type	3	22.73	0.28	3	18.94	0.64
Identity x tent type	3	21.97	0.30	3	7.41	0.88
Error	103	17.52		364	33.47	
Total	110			371		

The mean homozygosity percent in parent plants during 2020 was 85% compared to 77% of the progeny; progeny being significantly less homozygous by only 8% (Table 2). During 2021,

the homozygosity of both parent and progeny was remarkably similar (73%) without significance of difference.

The mean homozygosity percent among tents varied from 80 to 83% during 2020 and from 71 to 74% during 2021 with non-significant differences (Table 2). This is an important finding that while there was no differential response of tent types the genetic constitution of the parental family differed from its progeny with variable extent among different tent types especially in 2020. Overall, the data suggests that the differences between the tent architectures have not contributed to a tendency to cause breakdown in maternal sterility differentially, nor have the fabrics shown themselves differentially permeable to pollen from outside the PCT.

Table 2: Fitted means values for homozygous markers of the 209 total markers tested in parent and progeny covered with four types of tents during 2020 and 2021.

Term	Category	Mean homozygosity 2020 (%)	Mean homozygosity 2021 (%)
Identity	Parent	85.23±1.08	72.79±1.29
	Progeny	77.39±0.58	72.58±1.08
Tent type	DWB01	80.38±1.48	72.72±2.42
	DWB10	80.94±1.35	73.09±1.44
	DWB23	83.15±1.03	73.51±1.31
	DWB24	80.76±0.98	71.22±1.31

Evidence from molecular markers indicated all fabrics to be equally pollen-proof, and the parent CMS having residual heterozygosity unlike pure-breeding male sterile lines. We shall explore the consequences of residual heterozygosity on selfing and random mating later.

Seed Related Traits

A combined analysis of variance over two years (2020 and 2021) showed significant differences between covers and years for all traits. Interactions between years and cover types were significant for total seed weight, SW of >2.8mm seeds and implied number of germinated seeds (Table 3).

Table 3: *P*-values for significance of sources of variation in the combined analysis of variance for different seed-related traits over two years-2020 and 2021. Given in parentheses is the percent contribution of sum of squares of each source to the total sum squares (SS).

Source	DF	TSW (g)	SW >2.8mm (g)	1000-SW (g)	IS	Germ (%)	ISG
Covers	4	0.00 (52.4)	0.00 (60.1)	0.00 (41.6)	0.00 (33.4)	0.00 (59.6)	0.00 (53.1)
Control Vs other	1	0.00	0.00	0.00	0.00	0.00	0.00
Bet Others	3	0.10	0.49	0.00	0.85	0.04	0.83
Grp 1 vs Grp2	1	0.02	0.15	0.00	0.71	0.01	0.36
Remainder	2	0.87	0.87	0.90	0.72	0.49	0.99
Years	1	0.00 (14.3)	0.02 (1.9)	0.00 (20.2)	0.03 (1.1)	0.01 (6.00)	0.01 (4.0)

Covers x years	4	0.00 (23.3)	0.00 (25.5)	0.12 (6.3)	0.81 (34.4)	0.81 (1.3)	0.00 (21.2)
Error	40	(10.1)	(12.5)	(31.8)	(31.2)	(33.1)	(21.6)
Total	49						

P <0.05 is significant 5%; *P*<0.01 is significant at 1%; *P*>0.05 is non-significant. TSW =Total seed weight (g); SW= Seed weight (g) of seed >2.8 mm; 1000-SW= weight of 1000 seeds (g); IS= Implied seed number; Germ = Germination % after 10 days; ISG= Implied number of total germinated seeds. Grp1= DWB01 and DWB10; Grp2= DWB23 and DWB24.

The significance of cover treatments was largely attributable to the differential response of open pollinated control vs the four cover fabrics. This item was uniformly significant for all traits. What SS was left between four cover types was significant for 1000-SW and Germination percent only. It means that the cover fabrics did not differ for the remaining four seed traits. This result has practical implications. Further for both 1000-SW and germination (%) the significant difference was consistently attributable to (DWB01 + DWB10) Vs (DWB23+DWB24) comparison of fabrics. Mean values for 2021 were significantly higher than 2020 for most traits except for implied seed number (Table 4). The open pollinated control mean was generally manyfold higher than the mean of all covers; minimum increase of 83% being for 1000-SW. This is not unexpected as abundance of pollen in the open pollinated control situation would result in higher seed set than under pollen barriers. Conversely, the decrease of overall mean of all the cover treatments ranged from 45% for the 1000-SW to 96% for the ISG Table 4). Apparently, covering of plants with tents has adverse effect on the physiology of plants expressed in the reduction of performance for some seed traits. It is of interest to note that lower germination rates by 71% supported by the lower seed weight by 86% suggests that what is collected as "seed" is largely not true seed and hence the implied seed number weighted by germination (ISG) is the most appropriate indicator of 'true seed' which was reduced by 96%.

Table 4: Mean values for significant main effects. Given in parenthesis of control is the percent increase of control mean over mean of all bag type treatments.

Factor	Treatment	TSW (g)	SW >2.8mm (g)	1000-SW (g)	IS	Germ (%)	ISG
Bag	Control	54.73A (355%)	46.30A (668%)	16.10A (83%)	2948A (228%)	69.33A (293%)	2145A (2182%)
	DWB01	9.53BC	4.58B	6.84C	1070B	5.40C	5B
	DWB10	7.71C	3.59B	6.27C	833B	23.95BC	11B
	DWB23	14.96BC	7.14B	11.22BC	736B	23.09B	163B
	DWB24	15.90B	8.80B	10.78B	955B	28.19B	197B
	SE mean (±)	2.69	2.61	1.10	283	5.82	186
	LSD 5%	7.69	7.46	3.13	808	16.63	530
Year	2020	11.49B	11.23B	7.77B	1456A	20.99B	277B
	2021	29.64A	16.93A	12.71A	1161A	34.99A	732A
	SE mean (±)	1.70	1.65	0.69	179	3.68	117
	LSD5%	4.86	4.72	1.98	NS	10.52	335

Means that do not share a letter are significantly different. NS= non-significant. TSW =Total seed weight (g); SW= Seed weight (g) of seed >2.8 mm; 1000-SW= weight of 1000 seeds (g); IS= Implied seed number; Germ = Germination % after 10 days; ISG= Implied number of total germinated seeds.

The 1000-seed weight and 10-day germination (%) showed two clear classes of cover treatments. DWB23 and DWB24 fell together with higher 1000-seed weight, seed germination at 10-day and implied seed number at 10-day germination (Table 4). The germination (%) of seeds from DWB10, DWB23, and DWB24 was significantly higher than zero values as expected for non-seeds. However, the implied seed number at 10-day germination from all bag covers was not different from zero mean or non-seeds. This indicates that no bag type returned significantly viable seeds as would be expected from seed set on a CMS line. Clearly, all are safe covers returning almost zero germinated seeds. This conclusion is reinforced from SW of seeds >2.8mm being on par for all bag types and either non-significant or close to non-significance from zero number of such seeds. The germination from DWB01 is as good as zero but why DWB10, DWB23, and DWB24 returned significantly higher germination from zero? This is worth examination. Were all these germinated seeds maternal the result of favourable micro-climate within tents? However, it is not possible to conclude this from the available phenotypic data. Cover types significantly interacted with years for total seed weight, seed weight >2 mm, and implied seed number of germinated seeds (Table 3). What is the importance of these interactions can be known from their contribution to the total interaction SS? The percent contribution of interaction SS of various traits varies from 1.3 to 34.4 as compared to the high contribution of cover types (33.45 to 60.1) for characters exhibiting significant variation.

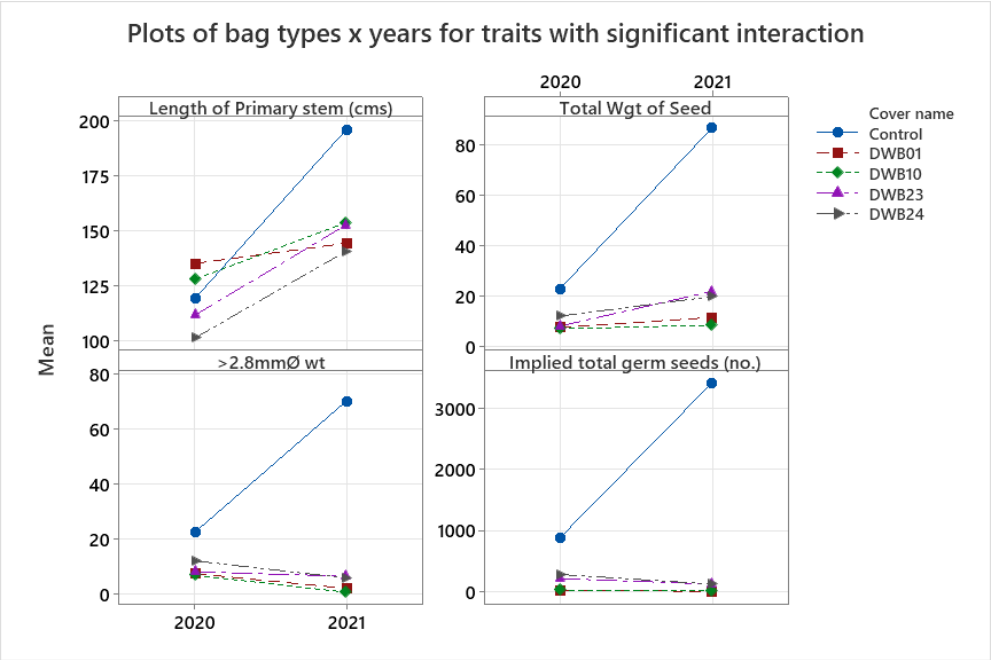

Figure 2: Interaction plot of bag types vs years for mean 1000-seed weight (g)

The contribution of years varied from as low as 1.1% to as high as 20.2%. It may be noted that when interaction SS contribution is less than the error SS to the total SS then the interaction effects do not demand a serious consideration. It may be concluded that generally interactions

though significant in some cases were not very important. Interactions have been presented in Figure 2 which reveals that low interaction for all cases largely resulted from the differential response of control over two years.

In general, all cover types showed lower performance than open control in both years indicating successful maintenance of cytoplasmic male sterility and exclusion of ambient pollen by all cover types. The differences between cover types in both years being very small suggesting it may not be worthwhile to use different cover types for different years. However, we are cautious that there were only two years in the present study and a study over more years could be more conclusive.

DISCUSSION

Sugar beet (*Beta vulgaris* L. ssp. v*ulgaris*) is the major source of sugar after sugarcane accounting for about one-quarter of about 160 MT world's sugar production annually [2]. It provides nearly 20% of the world's sugar production and is a major crop in Europe [28]. It also serves as a source for animal feed and feedstock for 30% of the bioethanol in Europe [24]. Almost all commercial cultivars of sugar beet are hybrids based on a three-line (male sterile, maintainer and restorer lines) cytoplasmic male sterility (CMS) system [5, 14, 18, 19],. Commercial hybrid seed production is achieved by growing CMS family along with a restorer family in an isolated field and letting the open pollination occur by wind and insects. Numerous experimental hybrids are generated during hybrid-variety development when artificial isolation is created through pollination control bags (PCB) or pollination control tents (PCT) for maintaining the genetic identity of each hybrid by disallowing unwanted foreign pollen to pollinate.

More recently, paper, cellulose or polyethylene pollination control bags (PCBs) are being replaced by more sophisticated designs made from nonwoven fabrics specifically developed for use with the particular biology of different plants. The standard PCBs, though cheaper, are prone to bird and weather damage. The new nonwoven PCBs are designed to be stronger, reusable, air permeable to support plant health, but retaining the essential isolation from external pollen [20, 21]. Several studies have shown that they return healthier and greater seed harvest in sorghum [9, 10, 25, 26, 27]; sugar beet, wheat, Arabidopsis and Miscanthus [7,12]; in grasses [1, 33]; in Indian mustard [11]; and in oil palm [4].

The nonwoven synthetic materials have very variable properties. Designing materials with maximum air permeability and light transparency helps to moderate the internal environment but simultaneously retaining pollen-proofing ability is a challenge. Application of a complex fibre architecture designed to capture pollen rather than allow passage is intended to compensate for pore sizes larger than the pollen grains of sugar beet. The aim is an optimal balance between pollen-proofing and air permeability.

The pollen-proofing ability of different fabrics is best tested by growing male sterile family plants, CMS in the present case, under different mini tents (PCTs). The extent of seed set under mini tents and the molecular fingerprinting of the progeny were hence used to identify its maternal or hybrid origin. Seed set on CMS plants could arise in two ways: (a) selfing resulting

from failure of male sterility or development of maternal seed by parthenogenesis [32], and (b) crossing due to ingress of pollen through the fabrics or rare mutations resulting in fertile progeny. While the progeny genotype from (a) will not differ from the genotype of the parental line, the progeny from (b) will differ from the parental genotype. However, both (a) and (b) assume the parental CMS to be pure breeding. When parental CMS family is heterozygous at some loci its selfing under isolation tents, assuming no pollen ingression, is expected to produce predictable genetic segregation in the progeny on the Mendelian model.

Gupta et al. [11] used KASPar marker assay for the fertility restoring gene *(Rfo*) to test the maternal or outcross origin of the seeds set on male sterile (MS) plants in Indian mustard. Invariably they found all progeny of maternal in origin (*rforfo*) under all synthetic fabrics' pollination control bags. Having pure-breeding male sterile line and specific fertility restoration gene was the most effective means of detecting outcrossing unlike the non-pure breeding male sterile family and non-availability of single fertility restoring marker gene in the present study on sugar beet. We employed multiple markers with more robust statistical analyses.

There was no difference in the homozygosity level of parent and progeny plants during 2021 since both showed 73% homozygosity. Also, all tent types behaved similarly with similar homozygosity levels. This result is consistent with no pollen ingression and that all fabrics exhibited pollen-proofing. However, molecular markers identifying homozygous loci during 2020 showed 85% homozygosity of the parent CMS family compared with 77% of the progeny unlike 2021 when both parent and progeny were 73% homozygous. The difference in estimated level of homozygosity percent in the two years could have been partly influenced because of the unbalanced progeny numbers which were much larger in 2021 with more seeds germinated and used for molecular analysis.

Molecular markers analysis over two years clearly showed that the CMS parent family was not genetically pure breeding and residual heterozygosity in the CMS family would segregate upon selfing or random mating. However, the extent of heterozygosity in this CMS family seems to have been accepted by sugar beet breeders since it is used in commercial hybrid breeding and seed production. There was 15% heterozygosity in 2020 and 27% in 2021 that is subject to segregation. This level of hybridity in the parental CMS ought to affect the outcome of progeny in terms of. the proportion of homozygous and heterozygous individuals under random mating or selfing depending upon gene frequencies.

The modelling of selfing series derived from F2 of a cross between two pure breeding parents is simple because of a biallelic situation at each locus where the alleles have predictable frequencies in any generation. However, this is not so for open pollinated populations due to unknown gene frequencies, the existence of poly-allelism, and non-random crossing. In a polymorphic population assuming a frequency of increasing A^+ and decreasing A^- alleles being p and q where $p+q=1$ the frequencies of genotypes in the population with random mating on a one gene model will be: $p^2A^+A^+ + 2pqA^+A^- + q^2A^-A^-$ [15].

One selfing generation caused by breakdown of male sterility or due to segregation of heterozygous loci in the tent will result in genotypic proportion of: $p^2A^+A^+ +2pq\ (1/4A^+A^- +1/2$

$A^+A^- + 1/4A^-A^-) + q^2A^-A^-$. This is equal to: $p(p+1/2)\ A^+A^+ + q(q+1/2)\ A^-A^- + pqA^+A^-$. Assuming equal gene frequencies (p=q=0.5) we have: 3/8 A^+A^+ +3/8 A^-A^- +1/4 A^+A^- = ¾ Homozygous + ¼ Heterozygous. This is the expected frequency of homozygotes and heterozygotes for a single gene locus from the selfing of an F2 with A^+ increasing and A^- decreasing alleles. The F2 genotypes are in ratio of: $1/4A^+A^+ + 1/2A^+A^- + 1/4A^-A^-$. The selfing of this population will result in F3 seed on CMS plants of which 75% will be homozygotes and 25% heterozygotes. The genotypic frequencies of F3 equivalent seed set on CMS plants in such a population resulting from outcrossing will become cumbersome. However, restricting to the simple model we expect 75% homozygosity and 25% heterozygosity among the progeny of seed set on F2 plants for segregating loci in the CMS family. Our observed estimate of homozygotes over two years was 75±0.50 % (77±0.58 % in 2020 and 73±1.08 % in 2021) in the progeny is not far from the expected when there could be superimposed effects of unequal gene frequencies, non-random mating and poly-allelism. We simulated the effect of gene frequencies on homozygosity levels in the progeny after one generation of selfing (Figure 3). The overall progeny heterozygosity levels over two years match with 0.5 gene frequencies and between 0.4 to 0.6 gene frequencies in the two years separately.

We can conclude from molecular markers that (a) The CMS family is not pure breeding; (b) the segregation in the family fits well with the genetic modelling of predictable outcomes from one self-generation of an open-pollinated population with no outcrossing; (c) All four tent fabrics are equally pollen-proof despite greater pore sizes of new fabrics compared to the standard.

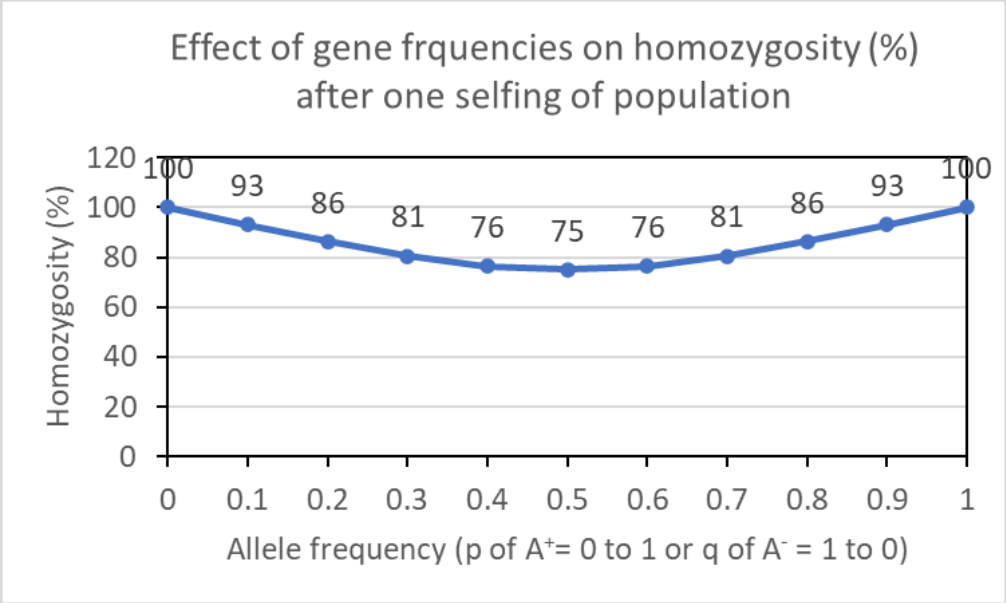

Figure 3: Effect of gene frequencies on homozygosity levels for biallelic single gene segregation following one generation of inbreeding of a random mating population

There was a non-significant difference between the four PCTs for all seed-related traits except 1000-SW and germination percent (Table 3). Despite significant difference for germination percentage, the mean implied germinated seed number (ISG) was a good fit to zero or non-seed for all cover types. Thus, no bag type returned significantly viable seeds as would be expected

from no seed set on a pure-breeding CMS line under PCTs. Further, mean values of SW of seeds >2.8mm for all PCTs were on par and either non-significant or close to non-significance from zero for various PCTs. Clearly, as for molecular markers, all covers are pollen-proof since they returned almost zero germinated seeds.

The purpose of the inclusion of open-pollinated control as treatment was to assess the abundance of pollen in the field. The control performed much higher for all traits over the PCT treatments, and its increase ranged from 83% for 1000-SW to 2182% for ISG (Table 4). Consequently, the significant treatments effects were the result of the high performance of open pollinated control for all seed-related traits resulted (Table 3). However, the four cover types showed no significant difference among them for most traits except 1000-SW and germination percent. For some agronomic traits other than seed-related traits (not reported here) Townson et al. [31] reported significant shading effect of PCT covers on performance of CMS plants. Plants grown in shade often tend to grow taller than they would grow outside under full sunlight. However, this is at the expense of energy and resources that could result in thinner main stem with fewer leaves or weaker roots and lower seed amount [16]. However, the lower performance of seed-related traits in the present case could not be wholly attributed to the shading effect but also to the non-availability of pollen inside the PCTs. An important conclusion from this study is that all new nonwoven PCTs were as effective in pollen-proofing as the standard DWB01. There was no significant difference in contamination by foreign pollen even when the new materials had pore size greater than the average pollen size of ~20-25 µm in sugar beet. Our conclusions are similar to Clifton-Brown et al. [7] who reported no pollen contamination on covered plants in sugar beet. This is the consequence of the complexity of the physical properties of nonwoven spun-bound fabrics where the pores create a torturous path through the fibrous mesh ensuring an uneasy passage for the external pollen infiltration. Wang and Gong [34] reported that the pore structure, pore size distribution, air permeability, and fabric area density of the 3D thermally bonded nonwoven filter samples consisted of multiple filtration layers of interconnected pores and tortuous pore paths through the fabric thickness. As suggested by Clifton-Brown et al. [7] and Townson et al. [31] this torturous path provides a trade-off in pollination performance of different tents. Our results show an acceptable filtration level of pollen exclusion in the materials tested though Vogel et al. [33] suggested maximum pore size of PCB should be kept under the pollen size of the crop.

CONCLUSION

This study uniquely used 209 molecular markers and some seed-related traits to confirm the pollen-proofing ability of three new nonwoven synthetic fabrics (DWB10, DWB23, and DWB24) of pollination control tents (PCTs) having larger pores than the standard (DWB01) using a CMS family of sugar beet. Molecular markers detected non-significant differences between PCTs but the parental family exhibited residual heterozygosity. All the four PCTs returned near zero viable seed and did not differ significantly. Both methods confirmed the pollen-proofing ability of new fabrics to be as good as the standard.

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Authors' Contributions

Paul Townson conducted field and laboratory experiments at the Lion Seeds, Essex and supervisory support, planning of experiments and editing the manuscript. Piergiorgio Stevanato and Samathmika Ravi conducted DNA extraction and molecular marker assessment. D.S. Virk contributed to planning, statistical analysis and preparing the draft manuscript. Hannah Senior supported the overall research from time to time and contributed in planning of experiments and editing the manuscript. All authors contributed to the final preparation of manuscript.

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