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Article

# Introgression of the *Afila* Gene into Climbing Garden Pea (*Pisum sativum* L.)

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**Abstract:** The pea (*Pisum sativum* L.) is one of the most important crops in temperate agriculture around the world. In the tropics, highland production is also common with multiple harvests of nearly mature seeds from climbing plant types on trellises. While the leafless variant caused by the *afila* gene is widely used in developing row-cropped field peas in Europe, its use for trellised garden peas has not been reported. In this study we describe a pea breeding program for a high-elevation tropical environment in the Department of Nariño in Colombia, where over 16,000 hectares of the crop are produced. The most widespread climbing varieties in the region are ‘Andina’ and ‘Sindamanoy’, both of which have high-biomass architecture with abundant foliage. They are prone to many diseases, but preferred by farmers given their long production season. This plant type is expensive to trellis, with wooden posts and plastic strings used for vine staking constituting 52% of production costs. The *afila* trait could reduce these costs by creating interlocking plants as they do in field peas. Therefore, our goal for this research was to develop a rapid breeding method to introduce the recessive *afila* gene, which replaces leaves with tendrils, into the two commercial varieties used as recurrent parents (RPs) with three donor parents (DPs)—‘Dove’, ‘ILS3575’ and ‘ILS3568’—and to measure the effect on plant height (PH) and yield potential. Our hypothesis was that the *afila* gene would not cause linkage drag while obtaining a leafless climbing pea variety. Backcrossing was conducted without selfing for two generations and plants were selected to recover recurrent parent characteristics. Chi-square tests showed a ratio of 15 normal leaved to one *afila* leaved in the BC<sub>2</sub>F<sub>2</sub> plants, and 31:1 in the BC<sub>3</sub>F<sub>2</sub> generation. Selecting in the last of these generations permitted a discovery of tall climbing plants that were similar to those preferred commercially, but with the stable leafless *afila*. The method saved two seasons compared to the traditional method of progeny testing before each backcross cycle; the peas reached the BC<sub>2</sub>F<sub>2</sub> generation in five seasons and the BC<sub>3</sub>F<sub>2</sub> in seven seasons. This is advantageous with trellised peas that normally require half a year to reach maturity. Leafless garden peas containing the *afila* gene were of the same height as recurrent parents and, by the third backcross, were equally productive, without the high biomass found in the traditional donor varieties. The value of the *afila* gene and the direct backcrossing scheme is discussed in terms of garden pea improvement and crop breeding.

**Keywords:** *afila* trait; backcrossing; climbing garden peas; *Pisum sativum* L.; trellises

## 1. Introduction

*Pisum sativum* L. is a multi-functional species used for human consumption as a pulse for its dried seed (field peas) or as a fresh vegetable grown for immature grain (garden/English peas) or pods (snow/snap peas) [1]. Dry field peas are grown during the summer in Australia, Canada, China, India,

Eastern Europe and the Northwestern United States; while garden peas are found throughout the world during cool seasons of the year [2]. Vegetable peas have been bred for a variety of characteristics, especially those related to consumer quality (sweetness, color, hilum appearance), while dry peas are usually bred for their high yield and disease resistance [3]. Dry peas are also used as feed grain for animal consumption where other feed legumes are not viable, such as in Western Europe [4]. In this research we concentrate on the garden pea grown for semi-mature seed and on types that are popular for fresh consumption in the Andes Mountains of South America.

Fresh garden peas are found mostly in temperate latitudes, but can also be found at high elevations near the equator. In the sub-tropics, they are usually planted as early spring or late fall/winter crops in low to mid elevations, while summer peas are planted at northern latitudes [3]. Meanwhile, in tropical highlands, peas can be planted year-round. One area of high elevation with a tropical production of garden peas is in the Andes Mountains of South America (Colombia, Ecuador and Peru principally), where the crop is grown in highland valleys and on inter-Andean plateaus, many with rich volcanic soils [5]. Within Colombia, the department of Nariño grows a large area of peas (more than 16,000 ha every year), employing over 30,000 people in the production chain, making this vegetable crop one of the most economically significant items in the rural economy of the region with a value of over 150 M dollars per year. Additional production is found in the Boyacá-Cundinamarca highlands and, to a lesser extent, in other high-elevation regions (near or above 2000 m above sea level) such as Antioquia and Tolima within Colombia, with small areas in Peru and Ecuador.

The costliest input to garden pea production in the highland tropical areas mentioned above is the construction of trellis systems. Trellising is needed since most traditional varieties of highland areas are vining types with large leaves and heavy biomass plants that would not stand on their own. The more bush-type, small leaf area and high-tendrill varieties of garden or field peas are not widely grown in Colombia. The most popular varieties, rather, are full-leaf genotypes that require strong trellising. These peas are often planted twice in a year on a trellis, following or preceding crops of maize, climbing beans or vining fruits.

Trellises to produce garden peas involve high inputs in terms of wooden posts installed at the end and often several times in the middle of each 50 to 100 m of row. The posts are held together by wire at the top and often along the sides at one or two intervals along the height of the posts. Individual pea plants are then fastened to the top wire with nylon, plastic or fiber strings. The posts must be replaced whenever they rot, and the wiring must be installed every few years. This represents a high cost in labor and supplies. Stringing the pea plants to the trellis is also time consuming and laborious. All these costs limit the areas that can be profitably planted with garden peas. Gently sloped and fertile land is in short supply, but is needed for trellised peas.

The high investment made for trellising, up to 52% of costs of production, reduces the likelihood of moving peas into new plots and, as a result, they are usually grown without adequate crop rotation. Root rots, leaf diseases and sometimes erosion can get out of control. A lack of crop rotation also increases the chances of stem diseases and insect pests occurring. Furthermore, trellising makes pest control difficult except with a manual backpack sprayer carried up and down the rows, which is often the most contaminating, expensive and inefficient method of applying pesticides.

One way to eliminate the need for trellising or to minimize investments in trellises would be to have sturdier plant architecture and more tendrils per plant, a characteristic bred into many dry peas used in temperate countries [6]. Tendrils at the tips of pea leaves or in place of some leaflets allow plants in a row to support one another and keep off the ground [7]. Additional tendrils replacing all leaflets provide even greater support [8]. In the trellising system, plants with many tendrils hold onto the plastic strings more firmly than non-tendrill or low-tendrill pea plants and require less labor, wires and twining support to train the vines to the trellis system [9].

In a non-trellis system, high-tendrill peas can grow taller than their low-tendrill counterparts because of within-row and between-row plant-to-plant support. In field peas, the use of high-tendrill germplasm has increased productivity and lowered production costs [10]. High-tendrill varieties tend

to have low lodging, since this is based on tendrils characteristics and overall plant height (PH), with the goal of plants that can grow tall to put on biomass but will not fall over. Leaf shapes in peas vary from those with tendrils and leaflets, through intermediate types (semi-*afila*), to those with only tendrils (*afila*) instead of leaflets. In general, the more tendrils a plant has the lower the disease pressure or lodging of the plant [11].

To date there have been no *afila* pea varieties developed in the Andes region of South America. However, their use would be promising for reducing the high biomasses of commercial genotypes grown in an intensive trellising system. While the leafless variant caused by the *afila* gene is widely used in developing row-cropped field peas in Europe, its use for trellised garden peas has not been reported. In this study we describe a pea breeding program for a high-elevation tropical environment using the *afila* gene. We also indicate the *afila* trait's advantages in a trellis system used for the support of peas, as is common in Colombia, Ecuador and Peru. As the climbing plant type is expensive to trellis with wooden posts and plastic strings used for vine staking, the *afila* trait could reduce these costs substantially by creating interlocking plants as they do in field peas, therefore requiring less string, posts and wire.

The specific objectives of this project were: (1) to introduce the *afila* gene into two climbing-type garden peas for use in southern Colombia; (2) to use backcrossing and intensive hybridization without selfing for three backcross generations (to avoid linkage drag), but with a minimum of time invested per backcross by avoiding progeny testing; and (3) to evaluate the yield effect of this introgression on the climbing garden peas typical of highland South America. The abbreviated backcross method recovered the commercial traits in lines containing the *afila* characteristic. Backcrossing was conducted with the trellised commercial varieties 'Andina' and 'Sindamanoy' with climbing pea phenotype as recurrent parents (RPs) and three bush-type pea breeding lines having the *afila* trait as donor parents (DPs).

## 2. Materials and Methods

### 2.1. Parents and Hybridizations

Crosses were made using recurrent parents Andina and Sindamanoy both with full leaflets and long vines of the climbing type. These were hybridized with three donor parents: the variety 'Dove', and the breeding lines 'ILS3575' and 'ILS3568', both from the Andean Crops Research project of the University of Nariño. Among the donor parents for the *afila* gene, Dove is a winter pea with determinate architecture that is tolerant to cold temperatures and produces on average 2600 kg/ha of green pods, which is less than the production of indeterminate vine-climbing types. The breeding lines have not been yield tested, but were *afila* genotypes derived from crosses made at the University of Nariño. Among the recurrent parents, the variety Andina (1.7 to 1.8 m tall) was released by CORPOICA/University of Nariño in 2002, and has an indeterminate growth habit with white flowers. Its grain is round, smooth and green with a white-colored hilum. This variety flowers at 65 days after planting (DAP), can be harvested green at 28 days after flowering (DAF) and is ready for dry grain harvest at 155 DAP. Its green pod yield has averaged 6607 kg/ha while its dry grain yield was 1848 kg/ha in the same study [12]. The variety Sindamanoy is also indeterminate, but even taller (1.9 to 2.3 m) than Andina, requiring a strong trellis. As a climbing pea it has two or three stems per plant and yields an average of 36 pods per plant (PPP), with each pod being 6–8 cm in length and having six to nine seeds. This variety also has white flowers, a round grain and seed that is green and smooth with a white hilum. The seed weight is 33 g per 100 seeds and yields have averaged 6400 kg/ha of green pods and 1600 kg/ha of dry grain in a previous study [12]. Its adaptation is from 2300 to 2900 masl (meters above sea level) in the region known as the Nariño altiplano.

## 2.2. Planting Site

Pea crosses and plantings were made in the field at the Lopez Farm (latitude 1°12' N and longitude 77°15' W) owned by the Servicio Nacional de Aprendizaje (SENA) in Pasto, Colombia, which is at 2633 masl and has an average temperature of 13 °C and annual total precipitation of 700 mm. The rainfall was bimodal and temperature did not vary during the year due to the equatorial location. Seed of the three crosses between recurrent parents (Andina and Sindamanoy) and the donor parents (Dove, ILS3568 and ILS3575) at the final BC<sub>2</sub>F<sub>2</sub> stage were planted in 20-m long plots of five rows, with 1.2 m between rows and 0.1 m between plants. For the following backcross generation, the BC<sub>3</sub>F<sub>2</sub>, was planted in the same way but with four rows each. The numbers of plants showing the two phenotypic states were then counted per row. Plants were categorized as either with the *afila* characteristic of tendrils replacing leaflets (representing a double recessive allele) or as plants with normal leaflets. A representative set of three segregant plants from each generation of backcrossing derived from the Sindamanoy × ILS3568 cross were grown to harvest to evaluate productivity in terms of number of pods per plant (PPP), pod length (PL), number of seeds per pod (SPP) and yield per plant (YLD) in grams (g/plant), as well as plant height (PH) in meters (m).

## 2.3. Statistical Analyses

Chi-square statistics [13] were used to estimate the goodness of fit to the expected backcross ratios for a single recessive gene occurring in homozygosity without selection in previous generations. The two ratios tested were 15:1 in the BC<sub>2</sub>F<sub>2</sub> generation and 31:1 in the BC<sub>3</sub>F<sub>2</sub> generation, on a per row basis. Morphological data were averaged and tested for correlations with the number of backcross generations. An analysis of variance for these averages (three generations, 2 *df*) provided a test of significance with thresholds of  $p < 0.5$  (\*) and  $p < 0.01$  (\*\*). Mean separation was performed with a Tukey's test for least significant differences ( $\alpha = 0.05$ ).

## 3. Results

Our hypotheses of goodness of fit were met for the BC<sub>2</sub>F<sub>2</sub> and BC<sub>3</sub>F<sub>2</sub> generations using chi-square tests, with the basis for expected and observed segregations, as shown in Table 1. In each case the chi-square values for average segregation ratio for the BC<sub>2</sub>F<sub>2</sub> were lower than the tabular chi-square values at  $p \leq 0.05$  (Table 2). In the Andina (recurrent parent) × Dove (donor parent) population averages of 174 plants were normal-leaved and 14 plants had the *afila* trait for the five rows; the observed chi-square value of 0.46 was lower than the expected value of 3.84. Meanwhile, in the Sindamanoy (RP) × ILS3575 (DP), similar results were observed, with 120 normal-leaved plants and six plants had the *afila* trait, for an observed chi-square value of 0.47. In the third cross of Sindamanoy (RP) × ILS3568 (DP), 109 plants had normal leaves and five showed the *afila* trait with an observed chi-square of 0.67. In all three cases, the null hypothesis of 15:1 segregation was accepted for the observed values compared to the expected values for the BC<sub>2</sub>F<sub>2</sub> generation.

For the BC<sub>3</sub>F<sub>2</sub> generation, the expected ratios were also confirmed. For example, a segregation of 158 normal plants and four plants with *afila* per row was observed in the population from the Andina (RP) × Dove (DP) cross, resulting in a chi-square value of 0.23 (Table 3). The cross of Sindamanoy (RP) × ILS3575 (DP) had a proportion of 174 normal plants and two with the *afila* trait and a significant chi-square value of 2.3. Finally, the Sindamanoy (RP) × ILS3568 (DP) cross showed the same tendency, with averages of 180 normal plants and three with the *afila* trait per row and a significant chi-square value of 1.32. All the observed chi-square values were lower than the expected value of 3.84 for a 31:1 ratio of non-*afila* to *afila* plants for the BC<sub>3</sub>F<sub>2</sub> generation.

**Table 1.** Abbreviated method for backcrossing the homozygous recessive *afila* gene (genotype *afaf*) into commercial varieties with normal leaflets (genotype *AfAf*).

Cross and Backcross Generations	Genotypes	Expected Parental Type Recovery	Season
Hybrid Cross	RP <i>AfAf</i> × DP <i>afaf</i>		Season 1
F1	<i>Afaf</i>	50% RP + 50% DP	Season 2
BC1 cross	( <i>Afaf</i> ) × ( <i>AfAf</i> )		
BC1F1	<i>AfAf</i> + <i>Afaf</i>	75% RP + 25% DP	Season 3
BC2 cross	( <i>AfAf</i> + <i>Afaf</i> ) × ( <i>AfAf</i> )		
BC2F1	4 <i>AfAf</i> + 2 <i>AfAf</i> + 2 <i>Afaf</i>		Season 4
BC2F1	3 <i>AfAf</i> + 1 <i>Afaf</i> ∅	87.5% RP + 12.5% DP	
BC2F2	3 (4 <i>AfAf</i> ) + <i>AfAf</i> + 2 <i>Afaf</i> + <i>afaf</i>		Season 5
BC2F2	15 <i>Af</i> : 1 <i>afaf</i>		
BC3 cross (from the BC2F1)	(3 <i>AfAf</i> + 1 <i>Afaf</i> ) × <i>AfAf</i>		Return to Season 4
BC3F1	3 (4 <i>AfAf</i> ) + 2 <i>AfAf</i> + 2 <i>Afaf</i>		Season 5
BC3F1	7 <i>AfAf</i> + 1 <i>Afaf</i> ∅	93.75% RP + 6.25% DP	
BC3F2	7 (4 <i>AfAf</i> ) + <i>AfAf</i> + 2 <i>Afaf</i> + <i>afaf</i>		Season 6
BC3F2	31 <i>Af</i> : 1 <i>afaf</i>		Season 6

RP = recurrent parent; DP = donor parent; ∅ = selfing event.

**Table 2.** Observed and expected segregation ratios and chi-square tests for numbered of plants with and without the expression of the homozygous recessive *afila* gene in the BC2F2 populations from the backcrosses made with the abbreviated selection method for three climbing × bush garden pea crosses.

Row	Observed Ratio		Expected RATIO	
	Without <i>afila</i>	With <i>afila</i>	Without <i>afila</i>	With <i>afila</i>
Andina × Dove				
1	191	18		
2	162	12		
3	170	13		
4	185	17		
5	163	11		
Average	174	14	15	1
Chi-square observed (X <sup>2</sup> c) = 0.46				
Chi-square test for p ≥ 0.05 (X <sup>2</sup> t) = 3.84				
Sindamanoy × ILS3575				
1	116	6		
2	111	5		
3	123	7		
4	128	8		
5	122	5		
Average	120	6	15	1
Chi-square observed (X <sup>2</sup> c) = 0.47				
Chi-square test for p ≥ 0.05 (X <sup>2</sup> t) = 3.84				
Sindamanoy × ILS3568				
1	90	3		
2	115	5		
3	102	5		
4	119	6		
5	120	6		
Average	109	5	15	1
Chi-square observed (X <sup>2</sup> c) = 0.67				
Chi-square test for p ≥ 0.05 (X <sup>2</sup> t) = 3.84				

**Table 3.** Observed and expected segregation ratios and chi-square tests for numbers of plants with and without the expression of the homozygous recessive *afila* gene in the BC<sub>3</sub>F<sub>2</sub> populations from the backcrosses made with the abbreviated selection method for three climbing × bush garden pea crosses.

Row	Observed Ratios		Expected Ratios	
	Without <i>afila</i>	With <i>afila</i>	Without <i>afila</i>	With <i>afila</i>
Andina × Dove				
1	195	1		
2	151	2		
3	155	3		
4	130	10		
Average	158	4	31	1
Chi-square observed ( $X^2c$ ) = 0.23				
Chi-square test for $p \geq 0.05$ ( $X^2t$ ) = 3.84				
Sindamanoy × ILS3575				
1	165	1		
2	164	4		
3	164	1		
4	204	2		
Average	174	2	31	1
Chi-square observed ( $X^2c$ ) = 2,3				
Chi-square test for $p \geq 0.05$ ( $X^2t$ ) = 3.84				
Sindamanoy × ILS3568				
1	221	1		
2	177	1		
3	207	5		
4	114	4		
Average	180	3	31	1
Chi-square observed ( $X^2c$ ) = 1.32				
Chi-square test for $p \geq 0.05$ ( $X^2t$ ) = 3.84				

All the backcross populations described above were created with an abbreviated method for backcrossing a recessive gene into a new commercial background, with no plant selfing performed on any of the backcross F<sub>1</sub> hybrid plants until the last cycle of backcrossing. Therefore, an F<sub>2</sub> was only generated for the last backcross hybrid. This procedure included BC<sub>2</sub>F<sub>1</sub> to obtain the BC<sub>2</sub>F<sub>2</sub> or/and BC<sub>3</sub>F<sub>1</sub> to obtain the BC<sub>3</sub>F<sub>2</sub>. The second generations were used to select the homozygous plants with the tendrilled phenotype and consequently the recessive homozygous *afila afila* gene fixed into a more commercial background, which would allow the development of a new improved variety containing the *afila* gene with a tendrill leaflet characteristic.

The chi-square tests showed the segregation of the *afila* gene as expected in the BC<sub>2</sub>F<sub>2</sub> and BC<sub>3</sub>F<sub>2</sub> generations, allowing us to discover a proportion of *afila afila* plants in rows of about 200 plants (Table 1). The method of direct backcrossing onto each hybrid generation without waiting for a self-pollination generation allowed the fixation of the *afila* gene in five seasons for the BC<sub>2</sub>F<sub>2</sub>-derived lines and seven for the BC<sub>3</sub>F<sub>2</sub>-derived lines. If a conventional scheme of selfing had been used to identify a few tendrilled, homozygous recessive *afila afila* BC<sub>1</sub> or BC<sub>2</sub> plants to cross with the number of seasons required would have been increased by at least two seasons, to seven and nine seasons, respectively.

Phenotypically, the backcross plants appeared more and more like the recurrent parent (Figure 1). With each generation of backcrossing, and as the genome contribution from the donor parent was lessened and the genome contribution from the recurrent parent was increased, we observed taller plants with the *afila* gene. An interesting aspect of this approach for converting the leafed non-*afila* peas to the leafless *afila* gene-carrying peas was that there was no linkage drag affecting climbing ability in the cross analyzed. Indeed, after three backcross generations, representative segregants returned

to the average height of the recurrent parent Sindamanoy of near 1.7 m, while in second and first generations of backcrossing they were significantly shorter as was the donor parent of *afila* (ILS3568) at only 0.7 m (Table 4). The F1 hybrid was 1.4 m, showing co-dominance with slight skewing towards the taller parent and indicating some role of dominant genes. By the BC<sub>3</sub> generation, climbing ability had returned to the level of the recurrent parent, being similar to the robust climber Sindamanoy.



**Figure 1.** Representative leaf morphology and plant heights for *afila* selections of garden peas, from three backcross generations of the Sindamanoy (recurrent parent) × ILS3568 (donor parent) cross shown at flowering stage. Twine shown at approximately every 0.4 m of vertical distance.

**Table 4.** Phenotypic trait measurements of plant height (PH), pods per plant (PPP), seeds per pod (SPP) and yield (YLD, in g/plant) in the parents and three backcross generations of the Sindamanoy (climbing recurrent parent) × ILS3568 (bush-type donor parent) cross with tendrilled, leafless, *afila*-containing BC<sub>1</sub>, BC<sub>2</sub> and BC<sub>3</sub> plants ( $n = 3$ ).

Generation	Name	PH	PPP	SPP	YLD
Parental	Sindamanoy	1.68 A	41.67 A	5.67 AB	71.48 A
Parental	ILS3568	0.74 C	17.07 B	6.17 A	49.90 B
Hybrid	F <sub>1</sub>	1.40 B	23.67 AB	5.70 AB	56.00 AB
Backcross 1	BC <sub>1</sub> F <sub>2</sub>	1.59 AB	29.00 AB	5.67 AB	61.82 AB
Backcross 2	BC <sub>2</sub> F <sub>2</sub>	1.65 AB	31.33 AB	4.90 B	70.03 A
Backcross 3	BC <sub>3</sub> F <sub>2</sub>	1.68 A	39.67 AB	4.90 B	71.85 A
Tukey's LSD ( $\alpha = 0.05$ )	n/a	0.253	24.43	1.26	19.01

Abbreviations: LSD = least significant difference; PH = plant height; PL = pod length; PPP = pods per plant; SPP = seeds per pod; and YLD = yield. Averages with different letters within a column represent significant differences with Tukey's mean comparison test ( $p < 0.05$ ).

In terms of the measures of yield potential also shown in the table of means, representative plants of each backcross generation had subsequently higher pods per plant (PPP) and yield (YLD), with an intermediate status of the F<sub>1</sub> and BC<sub>1</sub> generations. Although PPP returned to recurrent parent



levels slowly, YLD in terms of fresh weight seed production returned quickly to seed levels in the recurrent parent. The backcross plants were significantly closer to the recurrent parent by the BC<sub>2</sub> generation based on Tukey's mean comparisons. Return to recurrent parent phenotype was almost complete by the BC<sub>3</sub> generation, with similar phenotypes and additive responses to the recurrent parent genome contribution. A low percentage of donor parent genome contribution in the derived lines could explain the transgressive segregants observed.

Analyses of variance (ANOVAs) for the morphological traits in the Sindamanoy × ILS3568 backcross were significantly ( $p < 0.05$ ) or highly significantly ( $p < 0.01$ ) different among generations (Table S1) for all traits except pod length, for which they were not significant ( $p > 0.05$ ). Pearson's correlation between the number of generations of backcrossing and the morphological traits varied from 0.96 for PH, to 0.84 for YLD, 0.72 for SPP, 0.64 for PPP and 0.53 for pod length (PL), with all being highly significant ( $p < 0.01$ ). Because PL was not significantly different between generations, the averages are not shown in Table 4. However, PL was still useful for correlation tests. The number of seeds per pod (SPP) was an interesting trait because the backcross generations BC<sub>1 to 3</sub> were lower in value than the donor parent, while the recurrent parent and hybrid were intermediate, suggesting that combining the climbing ability with the *afila* trait may have some effect on the number of seeds that can be created per pod. Fortunately, this did not affect yield potential seriously, as it increased in each generation—from 56 g/plant in the F<sub>1</sub> to nearly 72 g/plant on average in the BC<sub>3</sub>—surpassing even the averages of the recurrent parent (71.5 g), as well as the BC<sub>1</sub> (61.8 g) and BC<sub>2</sub> (70 g) generations. This might be explained by the number of pods per plant increasing rapidly from 24 on average in the F<sub>1</sub> to 29, 31 and 40 by the BC<sub>1</sub>, BC<sub>2</sub> and BC<sub>3</sub> generations, respectively. Genetic correlations between traits showed yield per plant and number of pods per plant (PPP) to be positively correlated with plant height (PH) having  $r$ -values of 0.999 and 0.935, respectively. Negative correlations were found between PH and PPP ( $r = -0.833$ ), as well as between PPP and seeds per pod ( $r = -0.508$ ), with pod length not significant.

## 4. Discussion

### 4.1. Success of Abbreviated Backcrossing the *Afila* Gene

In this study, we were successful at backcrossing the *afila* gene for tendrilled leaves into two background cultivars, creating new populations segregating the trait using either two or three backcrosses. We monitored segregation ratios and found them to be as expected in each generation. Given this, no selfing was required to select for *afila* genotypes in the background of the Colombian varieties. Return to the recurrent parent phenotype was important in the backcrossing technique because of the specialized nature and trellised use of the tall garden pea landraces grown on expense-incurring posts and wires in Colombia. Backcrossing is the most frequently used method for the incorporation of a single gene into an adapted cultivar [14]. As such, the method is most popular for monogenically controlled traits, but can also be useful for oligogenic traits. Backcrossing is the recognized method for introducing a single gene, especially a recessive one, into an adapted cultivar where improvement of a Mendelian trait is required.

The backcrossing technique has been widely used in crop plants to bring in important architectural and other traits from foreign germplasm, not to mention many disease resistances. For example, the bush characteristic of *Cucurbita pepo* was introgressed into the 'Menina Brasileira' cultivar of the viny *Cucurbita moschata* L. pumpkin, to make a shrubby cultivar called 'Piramoita' [15]. Heat tolerance was improved in Malaysian chilli Kulai (*Capsicum annuum* L.) by backcrossing with AVPP0702 (*C. annuum* L.), a heat resistance cultivar [16]. Tetraploid oilseed *Brassica napus* was used to introgress male sterility to diploid *Brassica rapa* through backcrossing [17]. Restorer-of-fertility (*Rf*) allele in sweet pepper (*Capsicum annuum* L.) was transferred from hot pepper line AVPP9905 to several sweet pepper genotypes for cost-effective production of sweet pepper hybrid seeds [18]. Seed germination and emergence rate, fruit development, marketable yield, and postharvest fruit storage longevity was

improved in cucumber (*Cucumis sativus* L.) through backcrossing with chilling-tolerant US breeding lines [19]. These are just a few of the many examples existing in crop breeding programs where backcrossing has been used successfully for the transfer or monogenic or oligogenic traits.

In the case of garden peas, backcrossing has been used for architectural traits as was done here and also for various disease resistance genes. For example, Provvidenti and Niblett [20] used backcrossing of a single recessive gene to integrate the passionfruit woodiness virus into peas in North America. Backcrossing into the pea cultivar MK10 was used to introgress powdery mildew resistance controlled by the recessive *er1* locus [21]. A quality gene, *sin-2*, that controls fiber content and the stringless trait in pea pods has also been transferred through backcrossing [22]. *Fw1* and *Fw2* genes were integrated into garden peas through backcrossing for resistance to races 1 and 2 of wilt fungus *Fusarium oxysporum* f.sp. *pisi*, as well as *En* for resistance to pea enation mosaic virus and recessive *er1* for powdery mildew resistance [23]. In most of these cases, conventional backcrossing was necessary where self-pollination occurred after the BC<sub>1</sub>F<sub>1</sub> generation to determine if the gene was present and could be fixed into one of the BC<sub>x</sub>F<sub>2</sub> generations, with the fixed plant then used for further backcrossing. This ensured that the diminishing number of segregants with the desirable gene were actually present in BC<sub>3</sub>F<sub>2</sub> or the above generations [24]. In the traditional backcross method, plants with the recessive gene in homozygosity are crossed with the recurrent parent, resulting in heterozygous BC<sub>x</sub>F<sub>1</sub> and a 3:1 ratio of segregation by the BC<sub>x</sub>F<sub>2</sub>.

As seen from discussion above, conventional backcrossing relies on having a quarter of the plants express the desirable allele in a homozygous state by the F<sub>2</sub> generation, and for half to be in a homozygous state by the BC<sub>x</sub>F<sub>2</sub> generations, with a proportion of the recurrent parent genome dependent on the number of backcrosses. The main advantage of conventional backcrossing is that a plant breeder can generate a valuable BC segregant from a set of RP (RP × DP) crosses without generating large populations; however, waiting one extra season per cycle is necessary. This makes conventional backcrossing convenient for greenhouse work over multiple seasons in plants that are small in stature and do not take up too much space or require significant time to grow. On the other hand, the disadvantage for the conventional technique is found when the crop species or cultivar type is long-season or large in plant size, and therefore the extra time to reach an F<sub>2</sub> generation is a burden on the plant breeding program given that two cycles are needed—one for the BC<sub>x</sub>F<sub>1</sub> grow out and one for the BC<sub>x</sub>F<sub>2</sub> grow out. For this reason, we found a modified backcrossing approach to be useful for tall, late-maturing climbing peas.

The modification used was to abbreviate backcrossing by applying hybridization with the BC<sub>1</sub>F<sub>1</sub> generation when the ratio was 1:1 for the desirable allele, and then create multiple BC<sub>2</sub>F<sub>2</sub> and BC<sub>3</sub>F<sub>2</sub> populations in the following backcross generations. This method is most useful when a plant breeder has access to a large field and the chance to create the backcrosses in the field. For peas, the method was used before on a large backcross population generated for long-season, daylight-sensitive peas when selecting single-gene weevil resistance [25]. This technique was considered to save time and was an inspiration for the abbreviated backcross method carried out in our study.

#### 4.2. Abbreviated Method for Backcrossing of the Recessive *Afila* Gene

As mentioned above, when a trait is of simple inheritance and controlled by a single locus, backcrossing takes different forms when the overall field conditions vary. In addition, the conventional method is usually easier when the desired allele is dominant compared to the desired allele being the recessive one. Introgression of a dominant gene can often be done in the first filial generation of each backcross, while introgression of a recessive gene requires either selfing to a second filial generation or beyond, which is time consuming and constitutes the principal problem with traditional backcrossing. For example, in annual crops, the first backcross F<sub>1</sub> must be selfed to create an F<sub>2</sub> where the plants containing the recessive allele in a homozygous form are backcrossed to the commercial parent.

Conventional backcrossing adds to the number of seasons needed to create a new variety, especially as backcross cycles are added to the procedure. This makes backcrossing of recessive traits more

expensive and time consuming than transfer or dominant genes, where backcross  $F_1$ s can be crossed without selfing. Therefore, an abbreviated method is desirable for backcrossing, with alternatives mentioned above but especially in the context of introgression of a recessive trait, as was done in this study. In this case, the alternative to conventional methods is abbreviate backcrossing with the screening of consecutively larger segregating populations to detect the relatively lower number of recessive genotypes found in each successive backcross generation when no prior phenotypic or genotypic selection is practiced. This process can be referred to as the abbreviated backcrossing method and is the one we followed.

The abbreviated backcrossing method shown here is comparable to the transfer of a dominant gene in terms of the first generation of selection. Table 1 shows the inheritance of the transferred gene from the donor parents and the accumulation of background genes from the recurrent parents, as well as the segregation ratios for the dominant and recessive *afila* gene in the second and third backcrosses, namely, 15:1 for the  $BC_2F_2$  generation and 31:1 for the  $BC_3F_2$  generation. The abbreviated backcrossing procedure was successful for all three backcrosses made: Andina (RP)  $\times$  Dove (DP); Sindamanoy (RP)  $\times$  ILS3575 (DP); Sindamanoy (RP)  $\times$  ILS3568 (DP). As stated above, all crosses were made in the  $F_1$  generation without any advance to the  $F_2$  generation to make backcrosses, and multiple plants were used for crosses in the field rather than in the greenhouse.

The abbreviated method for backcrossing represents a significant time savings consisting of one season saved per cycle of backcrossing compared to the conventional method for backcrossing. For example, if four backcrosses are used to introduce a recessive trait into a commercial variety, the expected average recovery of the recurrent parent genome will be 98.875% and the savings in time to get to the  $BC_4F_2$  will be four seasons compared to the traditional method. The abbreviated method does require a greater number of crosses (at least 25 to 50) to be made, and therefore is more applicable to large-flowered species that are easy to cross, such as garden peas and common beans. Legumes are cleistogamous and this ensures selfing and avoids unwanted outcrossing. In the case of peas, about 40 crosses can be made per day, allowing crossing onto a population with lower allele frequencies.

#### 4.3. Utility of the *Afila* Gene for Introgression in Climbing Peas and Effect on Yield Ability

Among the most important results of our work was showing expanded geographic and agronomic use of the *afila* gene with no detriment to its use in climbing peas. From its origins in temperate countries of continental Europe and widespread use for bush-type field peas in the United Kingdom and North America, we show the promise of afileous peas in tropical South America that can grow at high elevations as long-season, trellised plants. As a well-characterized gene for increased tendrill production, *afila* has been recognized in peas for the past 60 years, but has mainly been used in Europe [26,27]. The *afila* gene transforms lateral leaflets on a composite pea leaf into tendrils instead of leaflets and is known to be of recessive inheritance [28,29].

The *afila* gene has also been evaluated physiologically in Australia, USA and India to date [30,31], and is known to interact with other genes for leaf morphology, including *unifoliata* and *cochleata*, showing even more extreme multi-tendrilled phenotypes [27,32,33]. One major element of the discovery and use of the *afila* gene was combining it with shorter statures using the Mendelian *le* gene for a more efficient architecture overall, much of which occurred behind the iron curtain starting in the 1970s in Eastern Bloc Europe [34]. Other pea breeding with the *afila* gene was done almost simultaneously in the United Kingdom, and resulted in releases such as the cultivars 'Sum' and 'Filby'. Continental European pea breeding with *afila* continued in France, Holland and Spain through the early 1980s with the release of commercial high-tendrill varieties such as 'Ascona', 'Ballet', 'Cea' and 'Elsa', all with yields similar to varieties with conventional foliage, many leaflets and a lower amount of tendrills [35]. Other varieties with the *afila* gene released by the private sector have included 'Solara' in 1986, 'Alex' and 'Choque' in 1989, 'Amadeus' in 1992 and 'Astuce' and 'Charleston' in 1993 [35], followed by 'Rampart' and 'Stampede' in 1990 and 'Golijat' in 2001 [7]. The Czech Republic also has had success in breeding afileous peas [36]. Expansion of the leafless pea trait to North America occurred when

breeders used *afila* and short statures from European germplasm to create field peas adapted to the Pacific Northwest. The first recombinants with the I/I genes for garden sweet peas were obtained at Cornell University by a researcher from Poland who had immigrated to the USA. In South America, a high-tendrill variety with the *afila* gene was released in Chile as 'INIA Brisca' and was developed from the cross of an individual selection named PS210791 × F2 (Porta × Neuga). No other South American *afila* peas have been released to our knowledge.

Modern field pea cultivars are very dependent on the *afila* gene, as they tend to be bush-type tendrilled architecture with low-leaf biomasses, but that are at the same time self-supporting. This both reduces foliar pathogens and plant lodging compared to older non-*afila* field pea varieties, as well as increases the air circulation in the canopy, reducing disease pressure on the foliage [35,37]. In addition, *afila* varieties generally have good overall productivity [36,38] and high water-use efficiency (WUE) compared to non-*afila* varieties [39]. A higher WUE and consequently better drought tolerance of *afila* peas results from the lower surface area of these varieties due to leaflets being replaced by tendrils, allowing for lower transpiration water loss than non-*afila* varieties [40]. While the benefits of drought tolerance and disease escape of *afila* peas were not part of our study, we did see that *afila* backcross lines can surpass the yield of non-*afila* peas, and they do so perhaps via larger seeds, despite having fewer seeds per pod. In our research, *afila* and non-*afila* breeding lines of climbing peas had similar number of pods per plant and plant heights, so yield differences were probably due to some seed physiology mechanism. Further studies can look at seed filling in the lines fixed for the *afila* gene. With climbing peas, this can be done at different heights within the canopy to determine the tendrils that photosynthetically contribute to pod and seed growth and development during the peas' life cycles. A further advantage of the lower leaf surface area in climbing peas that we worked with was that the planting density can be increased for *afila* varieties [41].

The physiological advantages and historical breeding achievements of *afila* peas were the inspiration for the work we carried out in this study. Our results on plant morphology and yield component trait analyses for characteristics such as pods per plant and harvest per plant suggest the polygenic control of both climbing ability and yield in relationship to height and growing period in the trellised garden pea varieties preferred in Andean production like Sindamanoy and Andina and the advanced lines generated by our breeding program. This contrasts with the monogenic inheritance of the *afila* trait even in the background of climbing-type peas. An interaction with seeds per pod seems likely independent of pod length. Furthermore, the multiple genes for climbing ability and yield found in peas [42] appear to be independent and unlinked with the *afila* gene for leaf loss and tendrill gain. If genes for climbing ability and yield had been linked to the *afila* locus, it would have been unlikely that the backcross progeny could recover the full climbing ability and pod yield of the recurrent parent varieties, which are vigorous climbers. Linkage drag was not observed with the *afila* gene in our analyzed cross, but has been seen in lodging studies with *afila* by Smitchger and Weeden (2019) [43]. Climbing ability and its effects on disease and lodging tolerance would likely be an additive trait with complex inheritance and quantitative trait loci (QTL). The combination of the *afila* gene in multiple backgrounds of garden peas seems very feasible, as it was for our two climbing garden pea parents. Furthermore, success can be achieved in three backcrosses.

In summary, we made crosses between *afila* bush-type peas as donor parents and tall (1.5 to 2.5 m), long-season (five to six month) climbing varieties as recurrent parents, and advanced the introgression of the *afila* gene into the trellised pea background. We were successful at selecting *afila*-type segregants after two to three backcrosses without using progeny testing, and thus we were able to accelerate the breeding process. Mid-parent heterosis of about 15% was observed for plant height but not for pods per plant or yield. Heterosis is usually not important in a self-pollinating crop like peas, but the additivity of yield component traits in the crop was promising for fixing background adaptation into advanced lines that are tendrilled rather than having leaflets. The taller the plant, the more yielding capacity it had, with taller plants having increased number of pods in each successive backcross. The easy introgression of *afila* was very important to our breeding program as well as to because of the high

investment needed to train and grow viny pea plants on trellises in research and production systems of the Andes. Farmers will certainly appreciate the new genotype once they realize that no yield sacrifice is found while realizing the potential savings to trellising efforts in terms of heavy extra work, wires and twining. On a further practical level, we obtained breeding lines that were equivalent to the recurrent parent in numbers of pods and seeds, but with the leafless trait and capability of climbing to 2 m or above. Since production potential, number of fresh pod harvests and total biomass of garden peas grown in the highlands is related to climbing ability, this is a very important discovery for breeding and agronomy of peas grown in the Andean region or similar parts of the world. The yield potential of the trellised peas with the *afila* gene will be studied in further studies now that the trait has been successfully introgressed into commercial-type breeding lines adapted to the highland environment, which was the major success of this research.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/10/1537/s1>. Table S1: Mean square values for five traits measured on two parents, F1 generation and three Backcrosses generations during the introgression of the *afila* gene into climbing garden peas (*Pisum sativum*).

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**Conflicts of Interest:** The authors declare no conflict of interest.

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