Analysis of growth and phenology in biotypes of *Raphanus raphanistrum* susceptible and resistant to ALS-inhibiting herbicides[1]

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ABSTRACT - The resistance of a weed to a herbicide may be accompanied by an ecological disadvantage, which can hinder the establishment of resistant biotypes when the herbicide is not used. The aim of this study, therefore, was to evaluate the growth and phenology of two biotypes of *Raphanus raphanistrum*, one susceptible and the other resistant to ALS inhibitors. The research was carried out from August to December 2020, conducting two simultaneous experiments, with the susceptible and resistant biotypes grown as single crops or together with wheat. The parameters under evaluation were plant height, number of leaves, leaf area, shoot dry weight, growth rate, leaf area ratio, net assimilation rate, and phenology – determined using the BBCH scale. The results showed not only that development and growth in the sensitive biotype was greater compared to the resistant biotype, but also the precocity of the biotype. The only variable for which the resistant biotype was superior was root dry weight. Cross-resistance to ALS inhibitors resulted in a difference in the growth and phenology of the resistant biotype that should be better evaluated, as the populations present large genetic differences.

Key words: Phenology. *Raphanus raphanistrum*. Competition.

INTRODUCTION

Since the beginning of agriculture and livestock farming, plants that spontaneously infested areas of human occupation and were not used for food, fibre or forage were considered undesirable (ZIMDAHL, 2018). To use the botanical term, such plants are considered pioneers, i.e. plants adapted by evolution to occupy areas where, for some reason, the original vegetation was profoundly altered, resulting in a large number of habitats for plant growth. Their function is to create habitats suitable for the start of a succession of populations that culminates in reestablishment of the original vegetation (PITELLI, 2015).

Weeds compete with crops of interest through a mechanism called interference, whose degree is influenced by factors linked to the crop (species, cultivar and plant arrangement), the weed community (specific composition, density and distribution), type of management (both for the crop and the weeds), and the period of coexistence between the crop and the weed community (time and duration), these factors being further dependent on the soil and climate conditions of the growth environment (BACHEGA *et al.*, 2013).

With the evolution of populations that are difficult to control, the adaptive value of resistant alleles may be accompanied by adaptive costs. In any given environment, these pleiotropic effects can be negative or positive, depending on the related allele (DÉLYE *et al.*, 2013). The competitive ability of resistant biotypes compared to susceptible biotypes – which often determines the proliferation of the biotype in the absence of a selective agent – mainly involves the ability of a plant to extract and accumulate nutrients, as well as their physiological performance (GALON *et al.*, 2013).

Costa and Rizzardi (2015) studied the growth of two biotypes of the wild radish (one resistant and the other susceptible to ALS inhibitors) in competition with wheat, and found similarities between both biotypes. In the literature, studies of the competition between biotypes of the wild radish and wheat predominate, leaving growth under competition-free conditions still to be determined. In Australia, Goggin *et al.* (2019) found no ecological impact from single resistance cultivation involving a biotype of *R. raphanistrum* resistant to auxin-mimicking herbicides.

In general, weed resistance to ALS inhibitors is not associated with any adaptive cost for the resistant biotype. Under competition-free conditions, biotypes of *Bassia scoparia*, resistant and susceptible to herbicides of the sulfonylurea chemical group, showed similar growth and seed germination. However, under interference, the biotypes showed a reduction in shoot dry weight that differed between the R and S biotypes,

with the R biotype being impaired by the interference (THOMPSON; THILL; SHAFII, 1994). A comparison of weed development in the absence of competition is essential for estimating the ecological adaptability of herbicide-resistant and herbicide-susceptible biotypes (CHRISTOFFOLETI; WESTRA; MOORE, 1997).

In earlier experiments to confirm resistance, a difference was seen in the initial growth of biotypes of *R. raphanistrum* that are susceptible or resistant to ALS inhibitors. These observations raised the hypothesis that resistance may have compromised the growth capacity of the biotype. The aim of this study, therefore, was to evaluate the growth and phenology of two biotypes of *R. raphanistrum*, one susceptible and the other resistant to herbicides that inhibit the ALS enzyme.

MATERIAL AND METHODS

Obtaining the material

Seeds of resistant (R) biotypes of *R. raphanistrum* were obtained from dose-response trials, in which cross-resistance to ALS-inhibiting herbicides was confirmed. This population came from the district of Catanduvas (25°21'26" S, 53°08'51" W and 752 m), in the western part of the state of Paraná; the seeds were obtained via self-fertilisation. The susceptible (S) biotype was collected in Lages, in the state of Santa Catarina, in a non-agricultural area with no history of herbicide application, located at the Centre for Agricultural and Veterinary Sciences (27°47'36" S, 50°18'08" W and 914 m), where mowing was only carried out to control weeds. For the S biotype, all the mature siliquas were collected from ten plants.

Conducting the experiments

The experiments were initially conducted in a greenhouse. Upon flowering, the experimental units were grown outside the greenhouse to facilitate fertilisation and keep seed production as close to natural conditions as possible.

The experimental design was completely randomised, with treatments organised in a 2 x 6 factorial scheme, in which factor A consisted of the R and S wild radish biotypes, and Factor B comprised the periods of evaluation: 12, 25, 50, 75, 100 and 125 DAE (days after emergence). Two simultaneous experiments were conducted with four replications. One experiment sought to evaluate the growth of each biotype in the absence of interference, i.e. free from the presence of other species in the same pot. In the other experiment, the biotypes were grown in pots together with wheat to simulate the interference that occurs in the field. Both experiments were sown and conducted during the same period, from August to December 2020, giving a total cycle of 118 days.

The experimental units consisted of plastic pots with a capacity of 3 dm³ that were filled with MecPlant® commercial substrate, and fertiliser equivalent to 400 kg ha⁻¹ of NPK 09-33-12 formulation. In the competition experiment, the TBIO Toruk wheat cultivar was used at a density of nine plants pot⁻¹, which is the recommended density for the crop, of 300 plants m⁻². In each of the experiments, the density of the wild radish was one plant per pot, the equivalent of an infestation of 30 plants m⁻².

Sowing was carried out simultaneously to simulate the natural growing conditions of the plants as much as possible. The wheat seeds were treated with Standak Top® (pyraclostrobin, thiophanate methyl and fipronil). The seeds of the wild radish biotypes were treated with insecticide and fungicide, in addition to Proggib® – active ingredient, gibberellic acid (40%) – at a final concentration of 5%. This plant hormone was used to break dormancy and ensure germination of the wild radish, particularly in the R biotype, whose seeds had recently been harvested. Once germination had stabilised (7 days after sowing – DAS), the experimental units were thinned to standardise the plant density.

Following the stabilisation of plant emergence at 7 DAS, the period began for evaluating the biotypes. In the experiment with the single crop, the evaluations started at 12 DAE, while in the competition experiment, they started at 25 DAE. To evaluate seed production, two treatments were carried out to the end of the cycle with the R and S biotypes grown with no interference.

The two experiments consisted of 24 treatments in 96 experimental plots. The treatments comprised a combination of susceptible and resistant biotypes with different periods of evaluation following emergence, representing growth up to that period. To evaluate seed production, treatments were maintained within any one experiment until the end of the cycle. The experiments were conducted under intermittent daily irrigation and constant maintenance to avoid attack by pests and diseases and prevent possible interference.

Evaluation and Statistical Analysis

Destructive tests were carried out to evaluate the morphology and phenological stage of the plants. Assessments were made of plant height, number of leaves, shoot dry weight, root system, reproductive structures (floral buds, flowers and siliquas), and of the entire plant. The dry weight was determined by oven-drying at 65 ºC to constant weight. The leaf area of the plants was also determined using a model LI-3100 bench-top area meter. Seed production was determined by collecting all the plant silica and by manual threshing. After threshing, the seeds were counted, and the weight of the seeds and the 100-seed weight were determined using a precision balance (0.001 g).

From the above data, we determined the performance of the biotypes using equations for growth assessment to calculate the relative growth rate (RGR), apparent assimilation rate (AAR), leaf area ratio (LAR), and leaf area index (LAI), this information being fundamental to better understand the growth of the biotypes.

$$RGR = \frac{LN(DM2) - LN(DM1)}{T2 - T1} \tag{1}$$

$$AAR = \frac{(DM2 - DM1)}{T2 - T1} * \frac{LN(LA2) - LN(LA1)}{LA2 - LA1} \tag{2}$$

$$LAI = \frac{LA2 + LA1}{DM2 + DM1} \tag{3}$$

$$LAI = \frac{LA}{AP} \tag{4}$$

Equations used to evaluate growth. RGR: relative growth rate, AAR: apparent assimilation rate, LAR: leaf area ratio, LAI: leaf area index, DW: dry weight, T: time, LA: leaf area, AP: area of the pot.
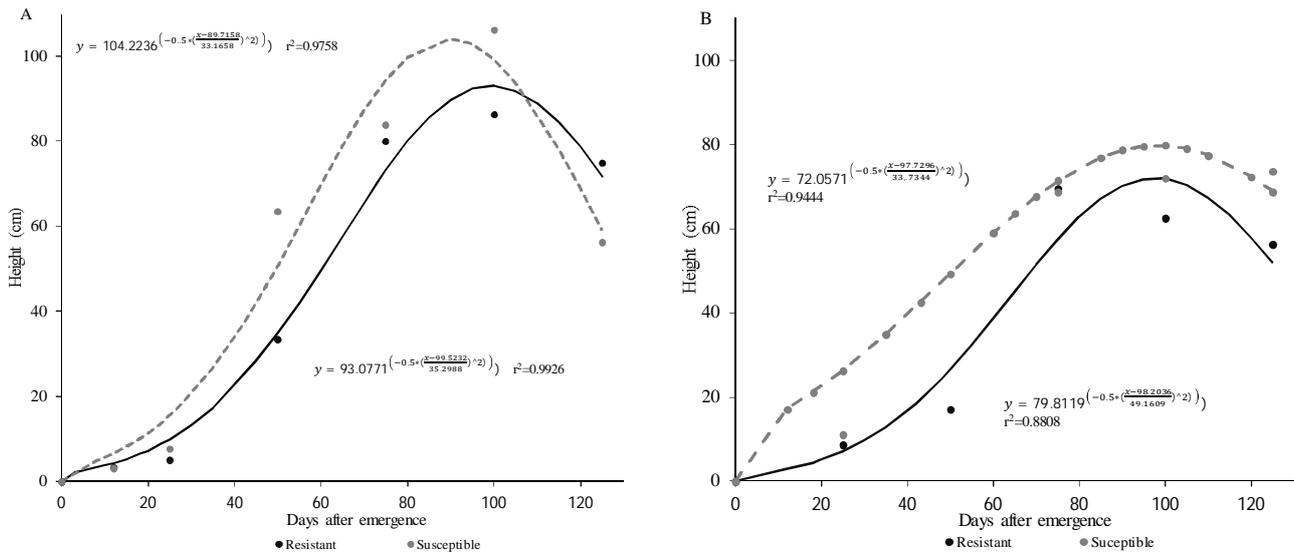
The above equations are used by various authors to evaluate plant growth, facilitating comparison with the literature; these equations were cited by Brighenti *et al*. (2001), who evaluated two milkweed biotypes, resistant and susceptible to ALS-inhibiting herbicides.

Variance and regression analyses were used to analyse the data. The regression equations were fitted to the Gaussian model (GUO, 2011). Seed production per plant, number of seeds per plant, and 100-seed weight were compared using Tukey's test at 5% probability.

Finally, the phenological stage of the plants was determined using the BBCH scale (HESS *et al*., 1997). The evaluations were made weekly, observing the development of the wild radish biotypes and correlating this with the respective phenological stage on the BBCH scale. Each of the biotypes was evaluated separately. The scale used in this study applies to several classes of plants, where each class has a different scale; in the case of the wild radish, the scale for weeds of class Magnoliopsida was used.

RESULTS AND DISCUSSION

There was a difference in growth and development between the two biotypes for each of the variables under analysis. A difference was seen in plant height (Figure 1): with no competition, the S biotype showed greater precocity and height, reaching more than 100 cm at 80 DAE (Figure 1), while the R biotype showed less growth, albeit with a longer cycle and maintaining its height for a longer period. The maximum height of the R biotype was 92 cm, and occurred at 105 DAE (Figure 1). Under competition, the differences were minimised, the growth of both biotypes being affected by interference from the wheat. Even so, the R biotype kept its competitive disadvantage.

Figure 1 - Height (cm) in resistant and susceptible wild radish biotypes, A: Single cultivation; B: Competitive cultivation

A

$y = 104.2236^{\left(-0.5*\left(\frac{x-89.7158}{33.1658}\right)^{\wedge 2}\right)}$ $r^2 = 0.9758$

$y = 93.0771^{\left(-0.5*\left(\frac{x-99.5232}{35.2988}\right)^{\wedge 2}\right)}$ $r^2 = 0.9926$

- Resistant   • Susceptible

B

$y = 72.0571^{\left(-0.5*\left(\frac{x-97.7296}{33.7344}\right)^{\wedge 2}\right)}$
$r^2 = 0.9444$

$y = 79.8119^{\left(-0.5*\left(\frac{x-98.2036}{49.1609}\right)^{\wedge 2}\right)}$
$r^2 = 0.8808$

- Resistant   • Suceptible

When evaluating a biotype of the milkweed (*Euphorbia heterophylla*) resistant to ALS-inhibiting herbicides, Brighenti *et al.* (2001) found no pronounced variation in plant height between biotypes, concluding that The R and S biotypes have similar growth and development, with no adaptive cost for resistance. Rampelotti *et al.* (2003) also found no marked differences in height for biotypes of *Sagittaria montevidensis* (resistant and selective to ALS-inhibiting herbicides). However, the authors made one important point: that in earlier observations such an effect existed and was visible, and that a more careful evaluation might confirm the adaptive cost. Mariani *et al.* (2016), evaluated the competitiveness to wheat of a ryegrass biotype resistant to ALS inhibitors, and reported that the R biotype was superior in height, but that the difference was not significant. These authors proposed that the adaptive value of the biotypes being studied was similar, and concluded that wheat has greater competitive ability than the biotypes under evaluation. In the present research, wheat was not evaluated, however, visual observations throughout the experiment revealed the broad competitive advantage of the crop over the wild radish.

The results for number of leaves (Figure 2) show a maximum value of 15 leaves for the S biotype at 60 DAE and 17 leaves for the R biotype at 75 DAE in the absence of interference; when grown together with wheat, these values were reduced, with a maximum of seven leaves in the S biotype at 55 DAE and 11 leaves in the R biotype at 65 DAE.

Unlike the results of this study, Galvan, Riszardi and Scheffer-Basso (2011) evaluated ryegrass biotypes resistant and susceptible to glyphosate and reported a variation in the number of leaves, where for two resistant biotypes, the number of leaves was higher, while for another resistant biotype the number of leaves was very close to that of the susceptible biotype. The same authors described the late senescence of the resistant biotype, and showed that this later senescence indicated a change in the plant cycle caused by alterations in the plant metabolism. In this respect the results are similar.

The biotypes also differed in relation to the increase in leaf area over time. With no competition, leaf area during the first 35 DAE was smaller in the R biotype compared to the S biotype (Figure 3A). The S biotype also had an earlier cycle, and maintained a larger leaf area for longer. However, the leaf area of the R biotype was larger, 747 cm² compared to 624 cm² in the S biotype (Figure 3A). The competition with wheat led to a loss of leaf area in both biotypes, albeit greater in the R biotype, with a maximum leaf area of 72 cm² compared to 206 cm² in the S biotype, i.e. a leaf area almost three times smaller (Figure 3B). In short, when grown together with wheat, the R biotype underwent a greater reduction in leaf area than did the S biotype, showing an ecological disadvantage from resistance.

Christoffoleti (2001) evaluated biotypes of *Bidens Pilosa* resistant and susceptible to ALS inhibitors, and obtained a greater leaf area in the S biotype, agreeing with the results of this study, where during the first 40 days of the cycle the leaf area of the S biotype was greater.

Unlike the behaviour seen in this study, when evaluating biotypes of *Conysa bonarienses* and *C. canadenses* resistant and susceptible to the herbicide glyphosate, Moreira *et al.* (2010) found differences between the biotypes. At the start of development, the leaf area of the resistant biotype was larger, however, over the course of the cycle the biotype began to develop more slowly, and its leaf area became smaller than that of the susceptible biotype.

Figure 2 - Number of leaves in resistant and susceptible wild radish biotypes. A: Single cultivation; B: Competitive cultivation
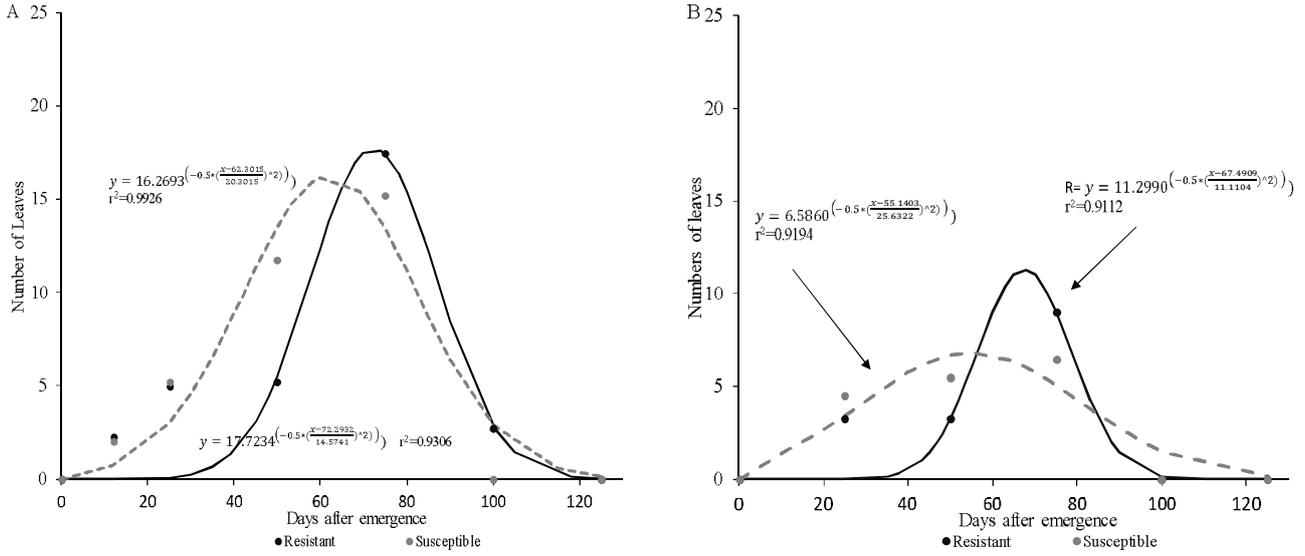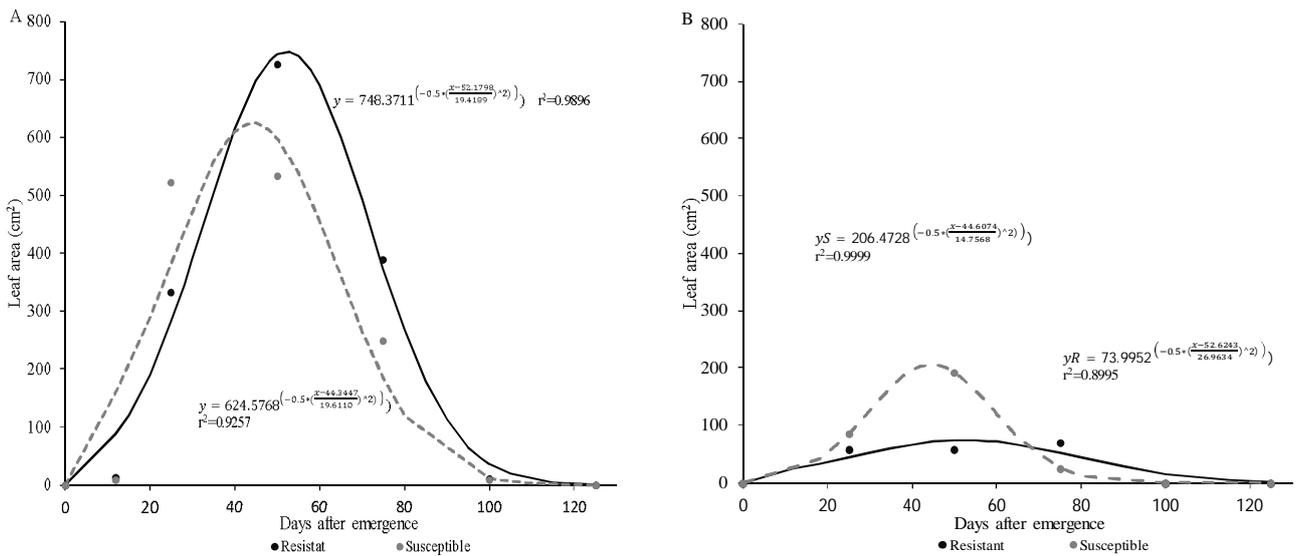

Figure 3 - Leaf area (cm²) in resistant and susceptible wild radish biotypes. A: Single cultivation; B: Competitive cultivation

There were similar differences in the values for dry weight (DW) (Figure 4). In the absence of competition, the S biotype was more precocious, reaching maximum DW accumulation earlier for each of the variables, and showing greater values for both shoot and total DW. On the other hand, root DW was greater in the R biotype. When grown under competition, this result was maintained, however, the R biotype suffered more from the process, showing a greater distance between the growth curves for shoot and total DW, and a smaller distance for root DW.

Tardif, Rajcan and Costea (2005), working with *Amaranthus powelli* biotypes resistant and selective to ALS inhibitors, also found that total dry matter production was lower and development was delayed in the R biotypes. Li *et al.* (2012) developed studies to investigate the resistance of wild radish biotypes in Australia; the R biotypes showed resistance due to a mutation in just one amino acid in the ALS enzyme. These authors found no effect on the growth of *R. raphanistrum* biotypes with resistance to ALS-inhibiting herbicides.

Figure 4 - Results for dry weight (DW in g) in resistant and susceptible biotypes. A: Total DW, single cultivation; B: Total DW, competitive cultivation; C: Shoot DW, single cultivation; D: Shoot DW, competitive cultivation; E: Root DW, single cultivation; F: Root DW, competitive cultivation
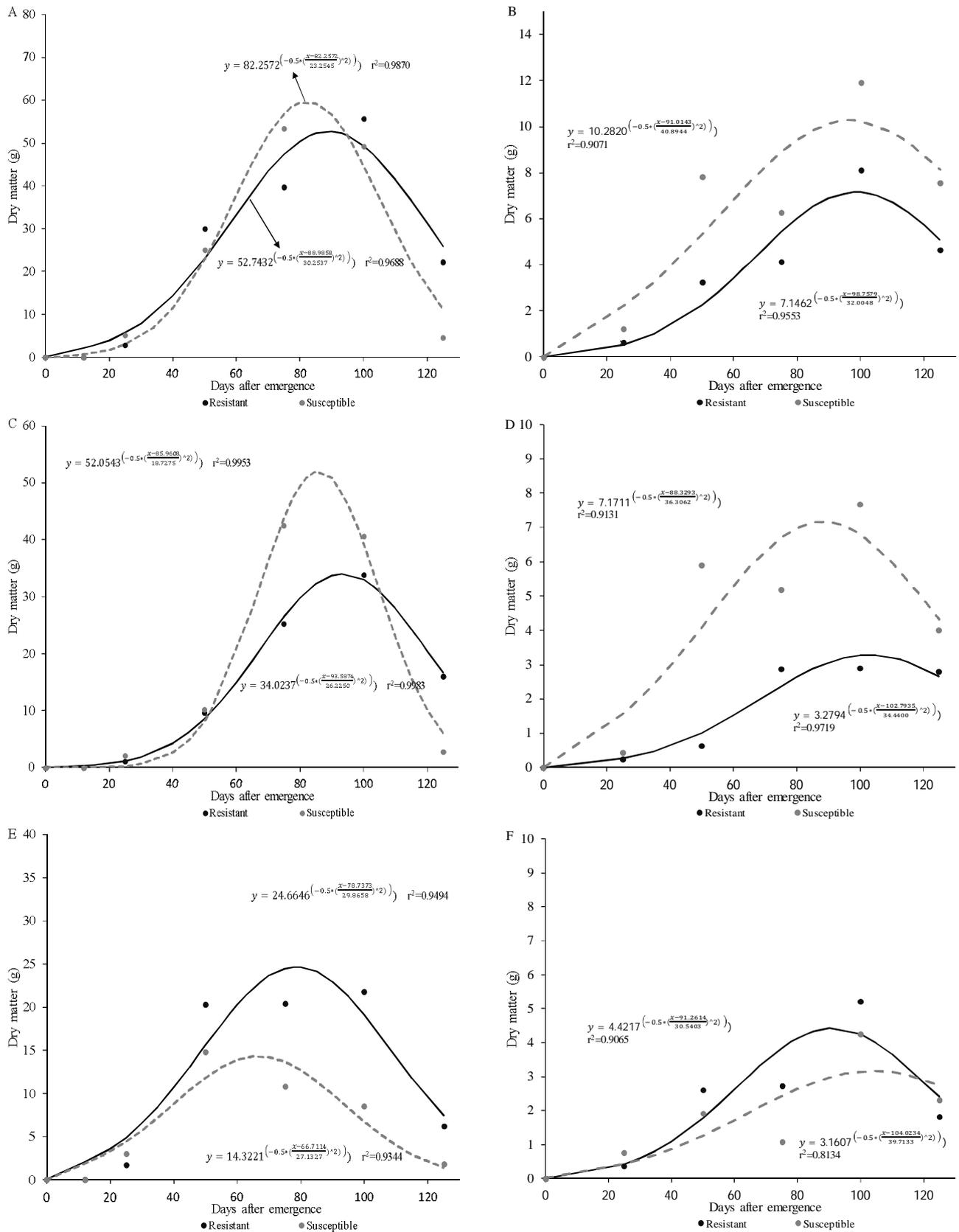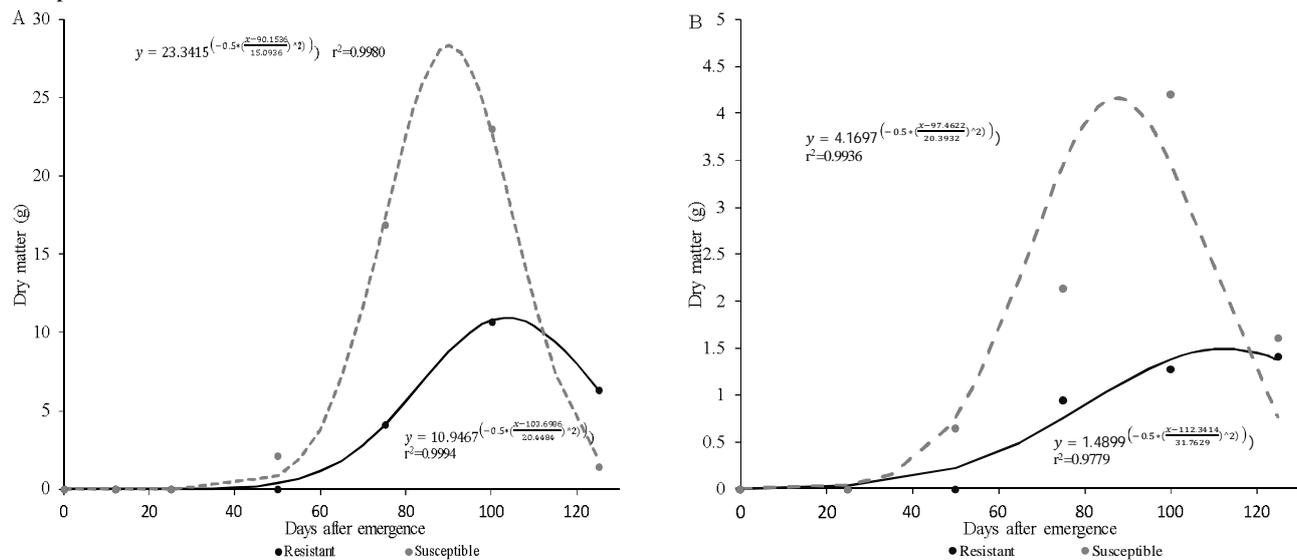
Figure 5 - Dry matter (g) of the reproductive structures of resistant and susceptible wild radish biotypes. A: Single cultivation; B: Competitive cultivation

The production of reproductive structures, silica and flowers was higher in the S biotype (Figure 5), this biotype producing in total twice as many reproductive structures as did the R biotype. The precocity of the S biotype was also maintained, with flowering and DM accumulation earlier than in the R biotype. Both biotypes were affected by competition, with the R biotype presenting few reproductive structures, and reaching a maximum of 1.5 g plant^{-1}, while the susceptible biotype achieved a maximum production of 4 g plant^{-1}.

The lower growth rate seen in each of the results, means that under normal conditions, without the presence of the selective agent (ALS inhibitor), the R biotype would show reduced growth and be subordinate in the population. When grown together with wheat at the plant density used in this study, the R biotype would be suppressed at a faster rate than the S biotype, resulting in a very low capacity for interference.
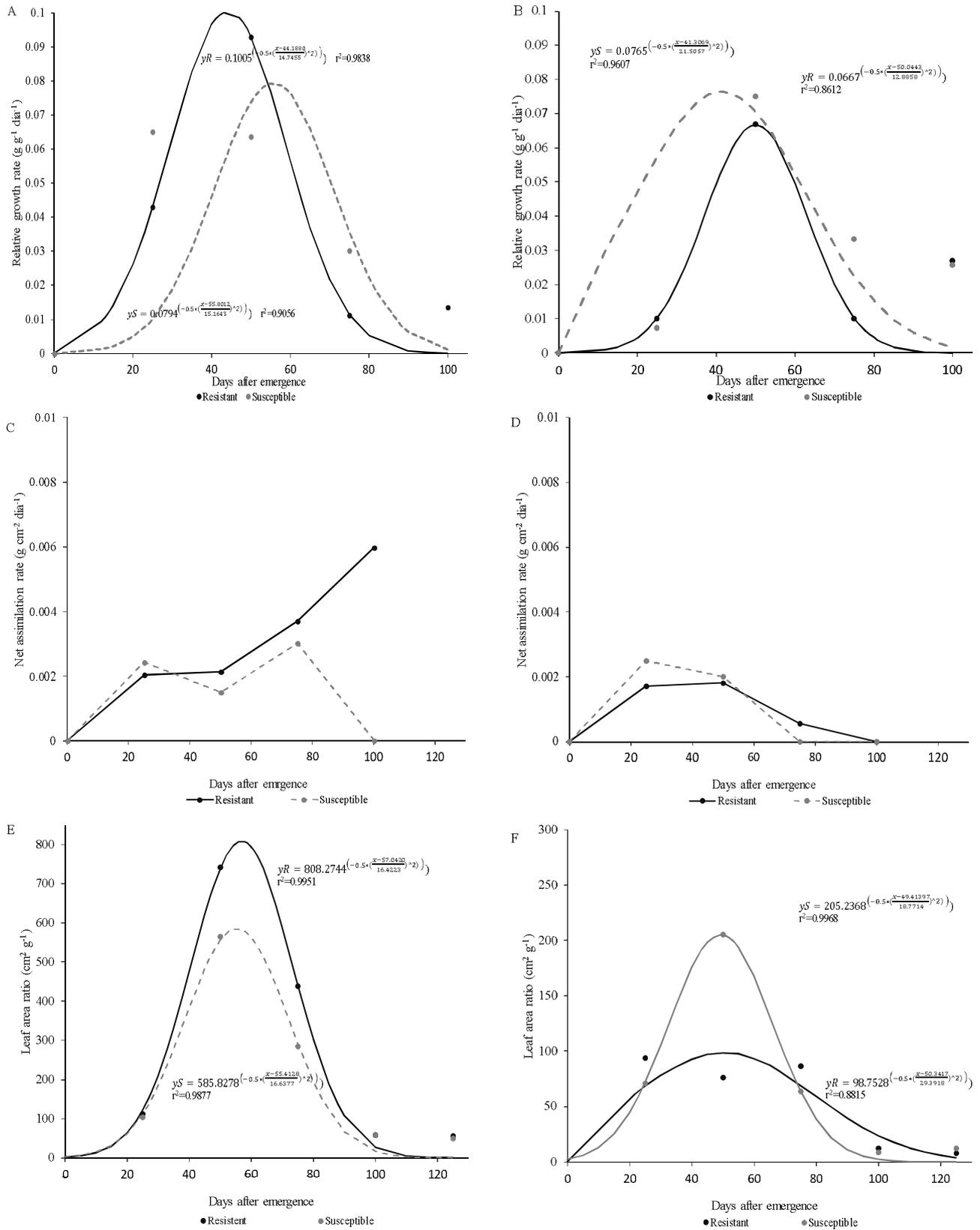
The results for dry matter of the reproductive structures are in line with the above results, the biotype being susceptible, with a far higher production of propagules. Production was greatly affected by competition, which reduced growth by approximately 90% between the two biotypes. The correct use of cropping practices benefits the crop in relation to the weeds, promoting reduced seed production, and consequently fewer additions to the soil seed bank, which is the main source of future weed infestations.

In a study with *Amaranthus powelli*, Tardif, Rajcan and Costea (2006) sought to verify the effects of mutation in ALS-inhibiting herbicides on the development and morphology of different biotypes, making

several statements, among them that the difference in competitiveness caused by the lower initial rate of development and the changes in morphology strongly reduce biomass accumulation and reproduction when R plants are grown under competition, as in the results of this study. This lack of competitiveness resulted in resistant plants producing far less shoot and seed biomass when competing with susceptible plants. Resistance to herbicides therefore appears to have a negative effect on ability in the absence of herbicides.

When grown free of interference, the R biotype showed greater relative growth (Figure 6A). This is probably due to better development of the root system (where the equation considers the total dry matter). Under interference, the relative growth rate reinforces the ecological cost of resistance, with a much higher rate in the S biotype, thereby reversing the results (Figure 6B). For the net assimilation rate, the biotypes were similar, with the exception of the evaluation at 100 DAE under interference-free conditions, when the R biotype maintained the increase in growth while the S biotype showed a reduction (Figure 6C and D). The leaf area ratio showed similar behaviour. When grown with no competition, the S biotype presented the largest area at 25 DAE, the ratio decreasing from that point on, whereas the R biotype presented the maximum value at 50 DAE, showing a more drastic reduction than the S biotype. Under competitive cultivation, the S biotype presented a higher leaf area ratio during almost every cycle, as well as a higher maximum value. On the other hand, the maximum ratio for the R biotype was twice as low, being more affected by the competition and accumulating less leaf matter and a smaller leaf area.

Figure 6 - Growth analysis of resistant and susceptible wild radish biotypes. A: Relative growth rate (g g⁻¹ day⁻¹), single cultivation; B: Relative growth rate, competitive cultivation; C: Net assimilation rate (g cm² day⁻¹), single cultivation; D: Net assimilation rate, competitive cultivation; E: Leaf area ratio (cm² g⁻¹), single cultivation; F: Leaf area ratio, competitive cultivation

Schaedler *et al.* (2013) evaluated biotypes of *Fimbristylis miliacea* with resistance to ALS inhibitors. Their results were similar to those of this study, where the R biotypes showed less development compared to the S biotypes, leading them to conclude an adaptive cost for the species.

Some substitutions that cause ALS resistance result in a reduction in ALS activity and substrate affinity. However, for other ALS mutations, there is no evidence of a reduction, including reports of no change in ALS activity (Pro-197-Thr) (PRESTON *et al.*, 2006). In the case of the mutation found in the present wild radish biotype (Trp-574-Leu), cases have been reported of a difference in biotype growth caused by the presence of the mutation. One example is the study developed by Tardiff, Rajcan and Costea (2005), who showed that several resistant biotypes of *Amaranthus powelli* with the Trp-574-Leu substitution exhibited slow development, reduced biomass and leaf area, and leaf distortion, compared to biotypes susceptible to ALS inhibitors. In the case of *Raphanus raphanistrum*, Li *et al.* (2012) showed that with or without competition for resources, resistance to the ALS gene via base-exchange mutations (Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu and Trp-574-Leu) has no negative pleiotropic effect on vegetative growth in resistant or susceptible plants.

For Vila-Aiub, Neve and Powles (2009), the mutation can also impair enzyme activity and/or reduce substrate affinity (resulting in insufficient product biosynthesis). Impaired ALS activity was found in the Pro-197-His substitution in *L. serriola* and *L. sativa*, and in the Ala-205-Val substitution in *So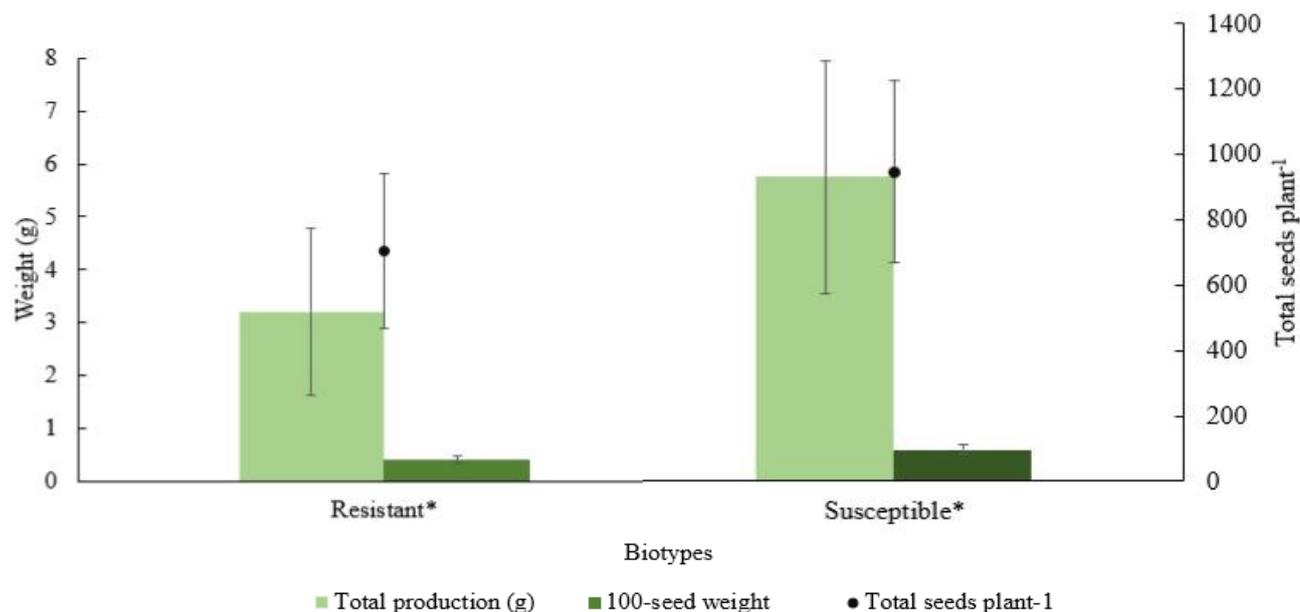lanum ptychanthum*. In both cases, the resistant ALS biotype showed no alterations in substrate binding, but an almost 50% reduction in activity compared to the S biotype (ASHIGH; TARDIF, 2007).

ALS activity is regulated by the accumulation of branched-chain amino acids (MIFLIN; CAVE, 1972). ALS mutations lead to reduced sensitivity to quantity inhibition by Val, Leu, and, or Ile. One possible consequence of reduced product sensitivity to inhibition is the accumulation of acidic branched-chain amino acids in the plant tissue. An excess and/or imbalance of the amino acid pool can have a toxic effect on cell metabolism, and correlates well with reduced plant growth (HOFGEN *et al.*, 1995).

Seed production differed between the biotypes. Despite no significant difference being recorded, on average, the S biotype showed greater seed production, number of seeds per plant, and 100-seed weight (Figure 7). A wild radish plant generates a large number of seeds that remain viable for a long period in the soil seed bank (MOROTA *et al.*, 2020).

The phenology of the wild radish biotypes, determined using the BBCH scale, demonstrates the difference between the life cycle of the biotypes (Table 1). The main difference is that the cycle of the R biotype was longer, with each of the phenological changes occurring later compared to the S biotype. At the start of the cycle, the principal difference was a lengthening of the rosette stage, which in the R biotype took more time, giving a difference of nine days between biotypes; for silica-drying, the difference was seven days. With the disadvantage of a later cycle, plants of the R biotype in a population of *R. raphanistrum* tend to be suppressed.

Figure 7 - Assessment of seed production in resistant and susceptible wild radish biotypes

* not significant by Tukey's test at 5% probability

Table 1 – Cycle and phenological stages seen in wild radish biotypes

		Susceptible				Resistant
DAE	BBCH	Stage	DAE	BBCH		Stage
		Seeding				Seeding
0	0	Emergence	0	0		Emergence
9	1	Leaf development	13	1		Leaf development
16	2	Formation of side shoots	16	2		Formation of side shoots
22	3	Lengthening of the rosette	29	3		Lengthening of the rosette
24	4	Development of propagative parts	31	4		Development of propagative parts
27	5	Inflorescence	34	5		Inflorescence
45	6	Flowering	54	6		Flowering
55	7	Fruit development	60	7		Fruit development
76	8	Ripening of the fruit and seeds	83	8		Ripening of the fruit and seeds
100	9	Senescence	108	9		Senescence
		1 – Leaf development				
1	10	First leaf emerged from the coleoptile	1	10		First leaf emerged from the coleoptile
12	11	First true leaf	12	11		First true leaf
14	12	Two true leaves, pairs of leaves or folded whorls	14	12		Two true leaves, pairs of leaves or folded whorls
16	13	Three true leaves, pairs of leaves or folded whorls	17	13		Three true leaves, pairs of leaves or folded whorls
25	19	Nine or more true leaves, pairs of leaves, or folded whorls	27	19		Nine or more true leaves, pairs of leaves, or folded whorls
		5 – Inflorescence				
24	51	Inflorescence or visible parts	27	51		Inflorescence or visible parts
25	55	First visible flowers	29	55		First visible flowers
27	59	First petals on the leaves	34	59		First petals on the leaves
		6 – Flowering				
27	60	First visible flower	34	60		First visible flower
43	65	50% of the flowers open	52	65		50% of the flowers open
52	67	End of flowering	57	67		End of flowering
55	69	End of flowering and visible fruit	60	69		End of flowering and visible fruit
		7 – Fruit development				
62	71	10% of the fruit at the final size	62	71		10% of the fruit at the final size
75	75	50% of the fruit at the final size	86	75		50% of the fruit at the final size
88	79	All fruit at the final size	96	79		All fruit at the final size
		8 – Maturation of the fruit and seeds				
70	81	Start of maturation	83	81		Start of maturation
81	85	Advanced maturation	100	85		Advanced maturation
104	89	Start of fruit abscission	108	89		Start of fruit abscission
		9 – Senescence				
74	91	Branch development complete, leaves still green	76	91		Branch development complete, leaves still green
75	93	Start of leaf fall	77	93		Start of leaf fall
85	95	50% fallen leaves	88	95		50% fallen leaves
100	97	End of leaf fall, plant or shoots dead or dormant	116	97		End of leaf fall, plant or shoots dead or dormant
118	99	Harvest	118	99		Harvest

Source: Prepared by the author, 2021

CONCLUSIONS

1. The results of this study showed that growth in the resistant biotype differed from that of the susceptible biotype. Under interference from wheat, this difference in growth is marked; however, due to the large genetic diversity between the populations, it is impossible to affirm any ecological cost, requiring further studies for this to be proved;

2. The BBCH scale, adapted for *Raphanus raphanistrum*, was an aid to understanding the phenology of the species.

ACKNOWLEDGEMENTS

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