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Resistance of a Local Ecotype of *Castanea sativa* to *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae) in Southern Italy

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Abstract: The cynipid *Dryocosmus kuriphilus* is the most impactful invasive pest of *Castanea sativa* copse woods and orchards currently reported from many European countries. A low impact solution for the containment of this pest could be the use of resistant trees. We examined the resistance of the red salernitan ecotype (RSE) of *C. sativa* to *D. kuriphilus* and carried out a morphological characterization of this ecotype's plants and fruits. From November 2015 to May 2017 we observed and recorded the percentage of infested buds, healthy leaves and shoots on about 50 chestnut trees, together with the number, size, and position of galls, and the number of eggs laid by the gall wasps into the buds and the number of larvae inside the galls. We showed a progressive mortality of cynipid larvae up to the starting point of galls development when almost total larval mortality was recorded. This suggests that RSE trees have a moderate resistance to *D. kuriphilus*; however, resistance acts at different levels, resulting in fewer eggs being deposited, a low number of larvae reaching the complete development, and a low number of galls on the branches. Moreover, the galls on resistant trees are smaller than the susceptible ones, so the larvae are more exposed to parasitization.

Keywords: Asian cynipid gall wasp; biological control; chestnut; invasive species; parasitization; resiliency; resistance

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1. Introduction

The Asian chestnut gall wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) is native to China and is harmful to all species belonging to *Castanea* genus (Fagaceae) [1]. ACGW is the only one of three chestnut gall wasps originating from the Palearctic region [2,3]; it has recently expanded its distribution area in Asia (Japan and Korea) and in a few years has reached both North America and Europe, becoming the key insect in chestnut orchards and forests of these territories [4–9].

Italy was the first infested European country; ACGW was first found in Piedmont in 2002 [10] then it reached Campania, where the first galls were found in May 2005 on young plants from Piedmont [6]. Since the first finding, ACGW has rapidly expanded its distribution area by affecting all chestnut areas in the rest of Italy over a few years [6]. ACGW was successively recorded in all European countries with important chestnut cultivations [8,11–14].

ACGW is a univoltine species, and its populations are composed entirely of females, which result from thelytokous reproduction that is not induced by bacterial endosymbionts [15]. Its infestation determines the formation of simple or compound galls that can be mono- or multilocular and develop principally on shoots (stem gall) and leaves. In the summer, females lay eggs into buds and larvae complete their development during the spring-summer of the next year when new adults emerge from galls [15].

Due to its concealed life cycle inside the chestnut buds and galls, the management of ACGW is challenging. Moreover, although there has been a quick shift of a very rich complex of indigenous parasitoid species from oak cynipids to *D. kuriphilus*, their action seems to be insufficient in controlling ACGW [16–20]. To date, the only effective method of control has been the introduction of *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), a parasitoid native to China [21–24]; the control effectiveness of *T. sinensis* reached satisfying levels after a variable number of years (6–18) [21,22,24–26].

Japan was the first country invaded by ACGW [27]. Before attempting to import a specific parasitoid, Japanese researchers tried to breed resistant chestnut varieties of the Japanese chestnut (*C. crenata* Siebold & Zucc.) and this strategy was successful for about 20 years. However, a novel virulent strain of *D. kuriphilus* overcame plant resistance [1,28], and the mode of inheritance of resistance was not established, which limits the application of modern methods for selection [1].

Hitherto, except the Italian cultivar Pugnenga (native to Cuneo Province (Piedmont Region)) and the French cultivar (Savoye) [29,30], all the cultivars resistant to ACGW belong to species different from the European chestnut (*C. sativa* Miller) (the Chinese chestnut (*C. mollissima* Blume), Chinquapin or dwarf chestnut (*C. pumila* (L.) Mill.)) or are hybrids with *C. crenata* [31]. In this latter case, they have acquired resistance by *C. crenata* (as in the case of the "Bouche de Betizac" cultivar) [1,30,31]. Italy has many indigenous cultivars appreciated all over the world for their organoleptic features, hence the lack of local resistant cultivars is cause for concern. About 50% of Italian chestnut production is in Campania (Southern Italy), with the Province of Avellino contributing to almost 60% of the regional production [32]. To date, no resistant local cultivars are known from Southern Italy that could be used both for replanting chestnut orchards and in reforestation programs. However, native chestnut germplasm in the South of Italy is widely diffused and individuating local chestnut trees with a high market value may be used both to promote a re-launch of chestnut cultivation and to preserve the native genetic resources. In this context, the problem raised by ACGW infestations led us to investigate the resistance of native cultivars to ACGW, in order to improve future actions aimed at planting chestnuts. Since the arrival of ACGW in Campania, there have been several reports of chestnut trees not showing galls, or whose level of infestation was rather low. At the end of 2015, in the Regional Forest of Roccarainola (Naples), numerous trees belonging to the red salernitan ecotype (RSE) that appeared to be resistant to the attack of ACGW were recorded, and their susceptibility was evaluated over two production seasons, along with a morpho-biological characterization of this ecotype.

2. Materials and Methods

2.1. Site Description

The study site is located in Fosso Agnone at 447 m a.s.l. (UTM coordinates: 33T 0462514 mE, 4,539,443 mN), within the 900-hectare Roccarainola Regional Forest. In this site, a chestnut orchard of 150 plants of about 20 years old covers two hectares. About 50 chestnut plants in the orchard were reported to be resistant to *D. kuriphilus* infestation. These grafted plants are randomly distributed among the other 100 susceptible trees (Figure 1) and are likely clones. Data collection started in November 2015 and lasted until May 2017. Samples were collected on both susceptible and resistant trees to make a comparison. Two releases of *T. sinensis* were carried out in 2012 and 2013 at this site; however, a two-year survey showed that *T. sinensis* was never found in this location [26].

Figure 1. Chestnut trees in Roccarainola study site: (**A**) susceptible tree; (**B**) resistant tree (marked with red paint) among susceptible trees (unmarked).

2.2. Morphological Characterization for the Identification of Plants and Fruits

Most of the susceptible chestnut trees belong to the Mercogliana cultivar, although the chestnut forest includes other local cultivars (Rossa di San Mango, Verdola, and Palummina). Non-susceptible plants and their fruits have been morphologically characterized by filling out cognitive data sheets, using internationally recognized agronomic and morphological descriptors (UPOV—International Union for the Protection of New Varieties of Plants) [33]. Phenological observations were performed on 10 plants by inspecting four branches at half crown height, two in the outer crown and two in the inner crown. In the summer, the phenological observations were made on fully developed leaves in the third median.

Samplings were also aimed at evaluating the carpological quality of the production. Fifty chestnut fruits were sampled to estimate the weight and shape, the hairiness at the base of the torch, the shape and size of the hilum, and the peelability of the episperm. A visual evaluation was carried out, following the methodology reported in the list of descriptors [33].

2.3. Evaluation of Resistance

Samplings involved buds, shoots, and galls.

2.3.1. Buds

The general resistance assessment in buds was made by a comparison of susceptible and resistant trees. Following the first inspection, in November 2015, when the absence of galls on RSE trees was confirmed, two samplings were conducted in February 2016 and 2017 (ACGW eggs of 2015 and 2016, respectively) to assess the number of hatching eggs and the presence of loci (gall chambers) and live larvae (at the first stage of development). In August 2016, to assess which type of resistance was involved (antixenosis, antibiosis, or tolerance) [34], a sampling was carried out to evaluate the density of ACGW eggs in the buds. During each monitoring, samples of 100 buds (ten replications, ten buds each, two shoots/tree, with 10 buds/tree) were collected from susceptible and resistant trees. Buds were checked externally for outward signs and then dissected under a microscope. Three parameters were evaluated for each bud: (1) the presence of scars; (2) the presence of laid eggs (August) or the presence of loci with live larvae (February); and (3) the presence of alterations in the internal bud tissues.

2.3.2. Shoots and Galls

Samples were collected on 10 different trees, 4 branches per tree, for a total of 2 linear meters per tree. The assessment of the presence of stem galls was made by evaluating 0.5 linear meters of each branch and collecting the following data: (1) number of galls (distinguishing galls developed on shoots or leaves); (2) number of healthy leaves longer than 2 cm; (3) number of healthy and infested shoots; and (4) mean gall size. Samples were collected randomly from susceptible and resistant trees, in May (2016 and 2017), with one branch collected per cardinal direction. After collection, two galls per branch (one leaf and one stem gall, when possible) were measured in height, width, and length (the diameter of each gall was obtained by calculating the average between these last two values). The mean gall size was calculated as in Bernardo et al. [15].

2.3.3. Evaluation of Percentage of Parasitization

A sample of 24 collected galls in May 2017 was dissected to observe the number and state of cynipids and parasitoids (all stages). The following formula was used:

$$\left(\frac{parasitized\ cynipids}{parasitized\ cynipids + healthy\ cynipids}\right) \times 100$$

A cumulative percentage of parasitization (all species together) was calculated because identification of immature stages of parasitoids (eggs, larvae, pupae) is currently almost impossible using morphological keys.

2.3.4. Statistical Analysis

Data satisfying conditions of normality and homoscedasticity, both untransformed or after appropriate transformation, were analyzed by ANOVA with the software Statgraphics Plus (Statgraphics Technologies, Inc., The Plains, Virginia, VA, USA) [35]. In all other cases, the Kruskal–Wallis *T*-test (KW) was used after having controlled for the data distribution to have the same shape [35]. The differences among the number of buds with scars, with eggs or larvae, and with alterations in the internal tissues, and lastly the differences in the percentage of parasitization of larvae on susceptible and resistant trees were analyzed by chi-square (χ^2) in contingency tables [36]. All data are presented non-transformed with their standard error (within brackets).

3. Results

3.1. Morphological Characterization for the Identification of Plants and Fruits

All resistant chestnut plants seemed to belong to an ecotype somewhat related to the local 'red' cultivar from Salerno, hereafter referred to as red salernitan ecotype (RSE). Characteristics of RSE plants and fruits are reported in Tables 1 and 2. RSE trees flower late and fruits, ripening late, begin to fall in the third week of October, about two weeks after the other local chestnut cultivars. The chestnut bur opens mainly along two valves, the mean number of chestnuts fruits for each bur is three and the mean weight of each fruit is 12 g, with 89 fruits weighing one kilogram. The pulp is crisp and has a sweet taste.

Table 1. Morphological plant characteristics according to the criteria classified by the International Union for the Protection of New Varieties of Plants (UPOV) [33].

Plant		Leaf		
Vigor (UPOV 1)	Habit (UPOV 2)	Size (UPOV 12)	Base Shape (UPOV 21)	Margin (UPOV 22)
Strong	Semi-upright	Medium	Obtuse	Acute

Table 2. Main carpometric characteristics of the fruits observed (n = 50) according to the International Union for the Protection of New Varieties of Plants (UPOV) [33].

Pericarp				
Color of Skin (UPOV 37)	Shape (UPOV 31)	Area of pubescence on upper part (UPOV 32)	Area of hilum (UPOV 33)	Shape of border line of helium (UPOV 34)
Reddish brown	Broad ovate	Medium	Medium	Straight
Seed				
Embryo (UPOV 28)	Degree of penetration of seed coat into embryo (UPOV 30)		Seed coat: adherence to kernel (UPOV 39)	Kernel: color of flesh
Mono-embryonic	Weak		Weak	White

3.2. Evaluation of Resistance

3.2.1. Buds

The scars of oviposition on perules (red and brown markings) were visible on RSE buds such as on those of the susceptible trees (Figure 2). Scars were more evident right after the deposition, in August (Table 3).

Figure 2. Red salernitan ecotype (RSE) buds after *D. kuriphilus* attack. (**A**) Scars resulting from the oviposition activity of Asian chestnut gall wasp (ACGW); (**B**) eggs of *D. kuriphilus* in dissected bud.

Table 3. Number of buds with scars, presence of eggs or larvae, and alteration in the internal bud tissues on a sample of 100 buds. X^2 contingency table tests.

Date of Sampling			Value	df	X^2	p
Scars	2.12.16	Susceptible Resistant	85 82	1	0.15	0.703
Larvae	2.12.16	Susceptible Resistant	94 73	1	14.52	0.001
Alterations	2.12.16	Susceptible Resistant	100 97	1	1.35	0.245
Scars	8.10.16	Susceptible Resistant	99 99	1	-	-
Eggs	8.10.16	Susceptible Resistant	97 88	1	4.61	0.032
Alterations	8.10.16	Susceptible Resistant	88 98	1	6.22	0.013
Scars	2.22.17	Susceptible Resistant	74 83	1	1.9	0.168
Larvae	2.22.17	Susceptible Resistant	97 88	1	4.61	0.032
Alterations	2.22.17	Susceptible Resistant	90 98	1	4.34	0.037

p = with Yates' correction, values <0.05 indicate significance.

Dissection of buds showed a very high percentage of infestation in both susceptible and resistant trees in both years of sampling (2016–2017) (Table 3). Moreover, the number of infested buds (by eggs or larvae) on RSE trees was significantly lower than that found on the susceptible trees in both years (Table 3). Differently, the number of buds with the presence of alterations in the internal bud tissues was significantly higher in RSE buds with respect to susceptible only in August 2016 and 2017 (Table 3). In August 2016, ACGW females laid a significantly lower number of eggs in RSE buds (17.6 ± 1.54) compared to susceptible buds (23.8 ± 1.51) (ANOVA test, $F_{1,198} = 11.49$, $p < 0.01$). Sampling carried out in February 2017 on buds showed the mean number of larvae/bud was 12.8 ± 0.82 for susceptible and 9.37 ± 0.55 for RSE (KW test, $t = 9.35$, $p < 0.01$).

3.2.2. Shoots and Galls

During the samplings, just a visual observation highlighted a difference between the branches of the RSE trees and those of the susceptible trees, with the former showing very few, if any, galls (Figure 3). In 2016, no gall on leaves were recorded on RSE trees, while 10.27 ± 1.171 galls on leaves/branch were recorded on susceptible trees. The number of stem galls found on RSE trees was very low compared to those found on susceptible trees (Figure 3) (KW test, $t = 54.69$, $p < 0.01$). In 2017, the number of galls on RSE trees was significantly lower than galls recorded on susceptible trees, both on leaves (ANOVA test, $F_{1,78} = 110.75$, $p < 0.01$) and shoots (ANOVA test, $F_{1,78} = 75.51$, $p < 0.01$) (Figure 3).

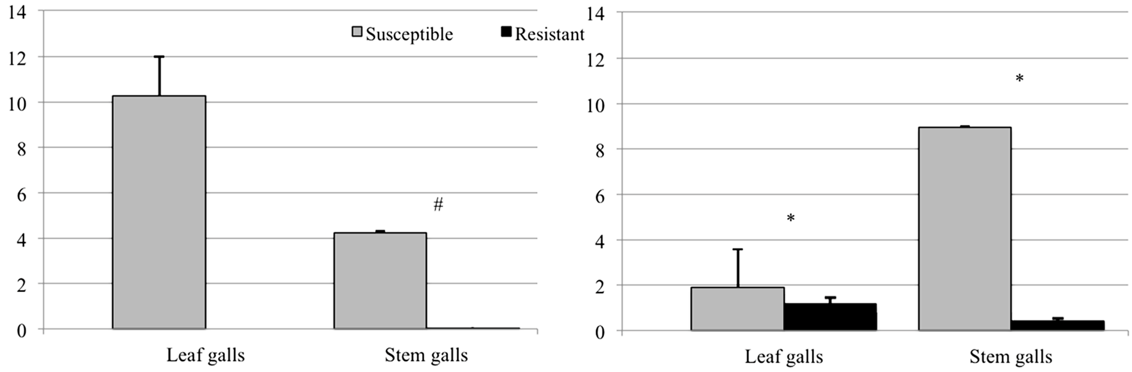

Figure 3. Stem and leaf galls on RSE and susceptible chestnuts in 2016 (**left**) and 2017 (**right**). Bars with # are significantly different at the 5% level, as determined by Kruskal Wallis test; bars with * are significantly different at the 5% level, as determined by ANOVA test.

The mean number of leaves per branch was very similar and no significant statistical differences were found in the comparison in 2016 (ANOVA test, $F_{1,78} = 0.66$, $p = 0.418$) and in 2017 (KW test, $t = 2.31$, $p = 0.128$) (Figure 4).

The mean number of shoots per branch was very similar and no statistical differences were found in both 2016 (ANOVA test, $F_{1,78} = 0.18$, $p = 0.669$) (Figure 4) and 2017 (ANOVA test, $F_{1,78} = 0.02$, $p = 0.886$) (Figure 4). The comparison of the number of healthy shoots per branch showed a lower number of healthy shoots on susceptible trees in both 2016 (ANOVA test, $F_{1,78} = 154.07$, $p < 0.01$) (Figure 4) and 2017 (ANOVA test, $F_{1,78} = 45.78$, $p < 0.01$) (Figure 4).

In 2016, comparison of the mean size of galls was not feasible due to the absence of galls on leaves and the low number of stem galls found (Figure 5). In 2017, leaf and stem galls collected on RSE trees were significantly smaller than galls collected on susceptible trees (Figure 5) (ANOVA test, $F_{1,33} = 35.27$, $p < 0.01$ for leaf galls; ANOVA test, $F_{1,57} = 38.40$, $p < 0.01$ for stem galls) (Figure 6).

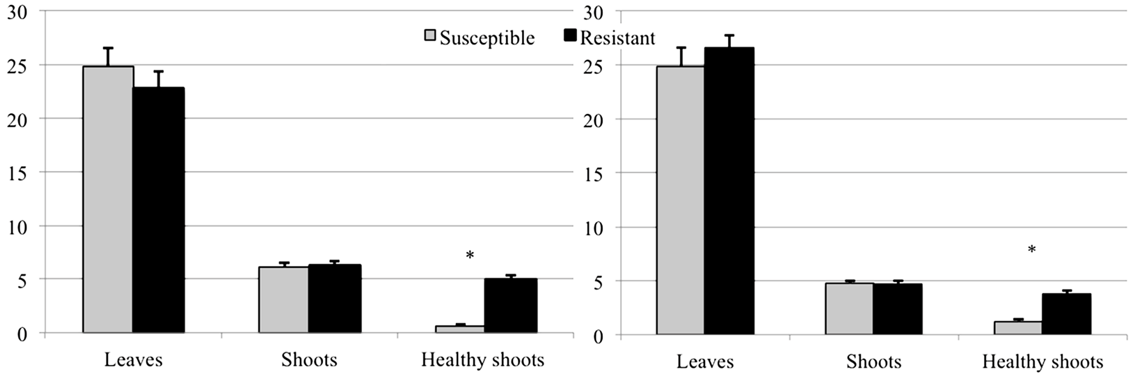

Figure 4. Mean number of leaves >2 cm, shoots (healthy and infested), and healthy shoots sampled on RSE and susceptible chestnuts in 2016 (**left**) and 2017 (**right**). Bars with * are significantly different at the 5% level, as determined by ANOVA test.

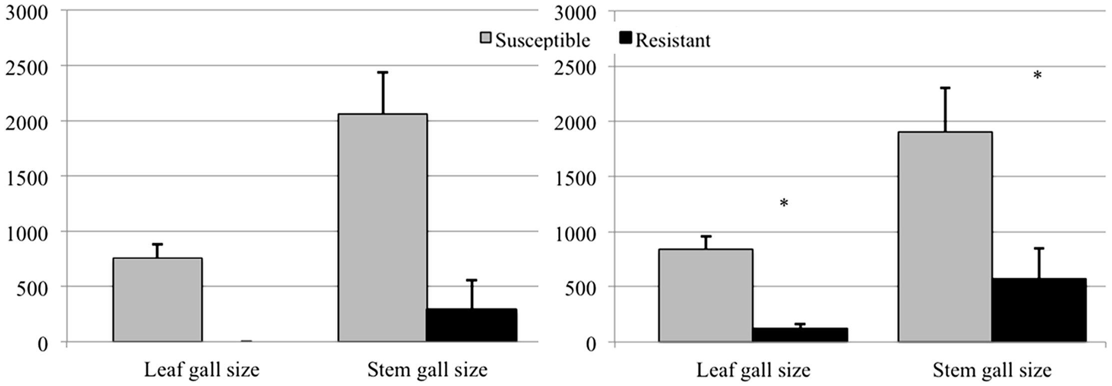

Figure 5. Mean size (in mm^3) of leaf and stem galls on RSE and susceptible chestnut trees in 2016 (**left**) and 2017 (**right**). Bars with * are significantly different at the 5% level, as determined by ANOVA test.

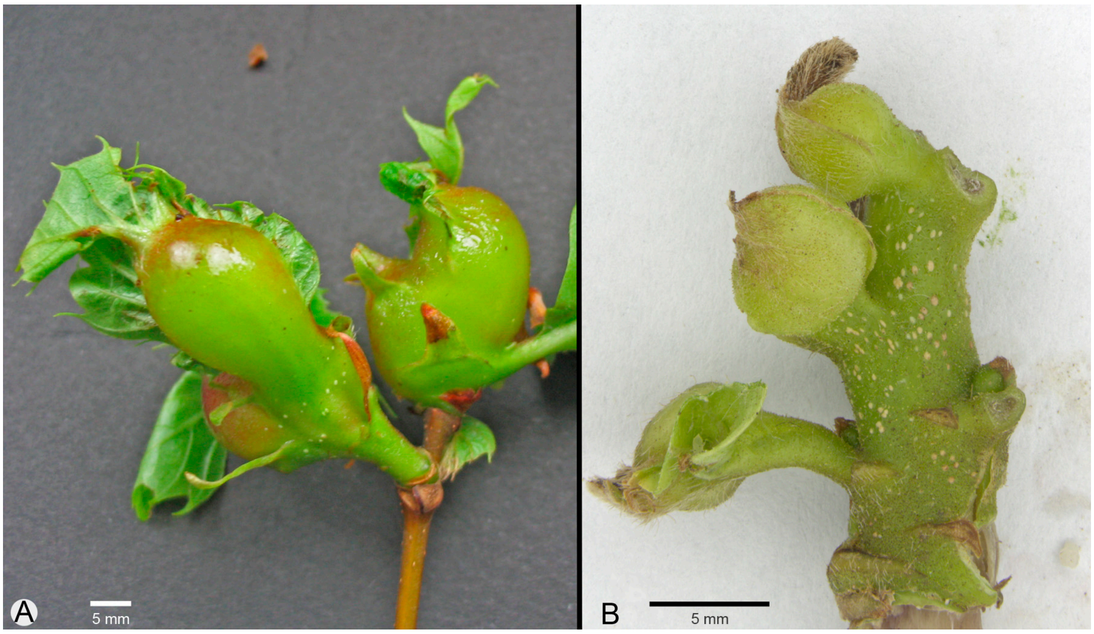

Figure 6. Stem galls collected on (**A**) susceptible and (**B**) red salernitan ecotype (RSE) tree.

3.2.3. Evaluation of Percentage of Parasitization

Percentage of parasitization was calculated only in 2017 due to the almost total absence of galls on the RSE trees in 2016. The rate of parasitization recorded on RSE trees was significantly lower than that recorded on susceptible trees (Table 4).

Table 4. Number of parasitized larvae in 24 susceptible and red salernitan ecotype (RSE) galls. X^2 contingency table tests.

Date of Sampling		Healthy Larvae	Parasitized Larvae	df	X^2	p
5.25.17	Susceptible	127	16	1	35.14	<0.001
	Resistant	77	59			

p = with Yates' correction.

4. Discussion

The red salernitan ecotype (RSE) has been shown to be moderately resistant to ACGW. Indeed, even if, as suggested by Panda and Khush [37], "not all resistance phenomena can be unequivocally assigned to one or other categories of resistance", the high plant-induced mortality of ACGW excludes that the observed phenomena are due to tolerance according to the definition of tolerance given by Koch et al. [38].

The resistance of RSE trees is not ascribable to a phenomenon of antixenosis because eggs and then larvae are present in the buds and galls. However, ACGW females lay a significantly lower number of eggs in the RSE buds. The RSE buds, therefore, seem to be less suitable for depositions than susceptible ones. RSE trees do not seem to produce volatile substances deterring ACGW females (from ovipositing), because no significant differences were recorded in the number of buds with scars between the two types of trees. Similar results were found in some cultivars of *C. crenata*, where no preference in oviposition choice between resistant and susceptible cultivars was reported [39]. Moreover, the presence of the same percentage of buds showing scars on perules, both in the susceptible cultivar and resistant ecotype, is a first indication that the RSE exhibits, at least in this phase of infestation, the same physiological reactions of the susceptible cultivars.

The red coloration and alterations in the internal bud tissues (small proliferated tissues) of RSE are identical to those found in the susceptible cultivars and wild trees [15]. This finding, together with the significantly higher number of buds with the presence of alterations (necrosis and/or proliferated tissues), confirms both the evidence of hypersensitivity symptoms just after the egg hatching and the occurrence of tissue modifications due to egg secretions soon after oviposition, as also reported for some oak cynipid species [40]. Evidently, in the first stage of infestation, plant tissues of susceptible and RSE trees do not react identically. Our finding seems to be slightly different from that observed in the Euro-Japanese hybrid "Bouche de Betizac", where plant cell necrosis occurred as soon as the eggs were laid [41]. For the above study, Viggiani and Tesone reported the presence of a reaction of hypersensitivity, which in fact, prevents the formation of the gall primordia causing an earlier ACGW mortality, concurrent with the hatching. However, other authors reported the hypersensitivity reaction in the same cultivar occurring after the larvae begin to feed and after the gall loci formation starts (just at budburst). Such a hypersensitivity reaction seems to be mediated by the presence of hydrogen peroxide [31].

Although our results cannot allow for an identification of the exact cause of resistance, we speculate that this phenomenon may be due either to the smaller number of eggs laid and to a different rate of larval mortality. In addition, some galls completed the development, and some larvae became adults in 2017 at a higher level of infestation. Based on these results, the underlying mechanism of resistance should be different from that found in the Bouche de Bétizac cultivar, where an early necrosis of the tissues was recorded [41]. The observed phenomenon is a moderate form of resistance because plants strongly affect the insect population [38]; indeed, although in low numbers, some ACGW larvae complete their development to adults.

Both susceptible and resistant trees are damaged by the depositions of ACGW (the percentage of bud infestation is very high) and by the subsequent development of some galls and necrosis in the buds. It is also worth noting that, even though all trees belong to the same ecotype and are likely clones, only some plants developed galls (a higher number in 2017 than in 2016). Hence there seems to be an individual variation of observed phenomena. Interestingly, galls found on RSE trees mainly affect shoots and are on average smaller, which may be due to the lower number of surviving larvae in the buds that cannot or do not need to modify a large amount of plant tissue. The smaller size of the galls and the involvement of less lignified tissues seem to have strongly influenced the parasitization. The larvae found in the galls on the RSE trees had, in fact, significantly higher parasitization than those on the susceptible cultivar.

Although we did not perform tests to evaluate the penetration resistance of galls, it is clear that the stem galls are on average larger, contain more chambers, and have a thicker sclerenchyma than leaf galls [15,42]. The smaller size of RSE galls makes all the loci (and the respective larvae) easily accessible to parasitoids, notably also making them more susceptible to parasitoids with short ovipositors that usually can parasitize only the larvae of the outer gall layers [42].

The presence of stem galls on RSE trees does not seem to influence the number of fully developed leaves. As can be seen in Figure 7, the distribution of fully developed leaves seems to indicate that the bud sector in which ACGW larvae live necrotizes while the remaining part develops normally.

Figure 7. Shoot with gall and leaves collected on red salernitan ecotype (RSE) tree (**A**); RSE chestnut fruit (**B**).

We did not investigate the mechanism that determines the resistance of RSE to ACGW, but it is possible that also in this case (as in the Bouche de Betizac trees) there is a hypersensitivity reaction that, as we stressed earlier, takes place later.

Recently, some Chinese authors have shown that the resistance of some *Castanea mollissima* cultivars is related to an increase in the production of phenylalanine ammonia-lyase (PAL), which is a key enzyme that catalyzes phenylpropanoid metabolism [43]. This information was added to that highlighted for some *C. crenata* cultivars resistant to ACGW in which the amount of catechol, pyrogallol, and total tannins is equal to those of the susceptible cultivars [44]. Overall, the mechanisms behind the resistance of chestnuts to ACGW are poorly studied and need further research.

The reported case of the resistance of RSE trees is the first well-documented case of resistance of a resistant local ecotype of *C. sativa*, as the previous reports of resistance were always related to other species of *Castanea* spp., with the single exception of the "Pugnenga" cultivar native to Piedmont [30,31]. This resistance is very interesting because, due to incomplete and gradual mortality of ACGW, it should not be easily overcome, differently from what has been recorded for the resistance of some Japanese cultivars in recent years [1,28]. This finding may be crucial for future genetic improvement programs, because it is easier to move a resistance between trees of the same species [45]. The use of trees of this ecotype, which is adapted to the climatic conditions of Southern Italy, could also integrate the action of *T. sinensis,* improving the control of ACGW in the long term. Lastly, the RSE trees produce good quality fruits, and the plants flower and fruit about two weeks later than almost all the cultivars cultivated in Campania (Figure 7). This tardiness allows for the avoidance of the frequent spring frosts, which have repeatedly damaged the chestnut production in Southern Italy in recent years.

5. Conclusions

The RSE trees are resistant to ACGW, given that the number of galls recorded on them is much lower than those found on the susceptible trees. Furthermore, the galls are smaller and therefore more parasitized. Moreover, the high quality of RSE fruits and its good and constant productivity makes it a good candidate to be used in reforestation programs in Southern Italy.

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