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in Forest Science

Effects of host trees on colonization patterns by ambrosia beetles under different abiotic stressors

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TABLE OF CONTENT

ABSTRACT

Ambrosia beetles are polyphagous insects able to attack several broadleaf trees species sometimes causing important damages. Most ambrosia beetles preferentially select trees in the early stages of physiological stress, using stressed-related volatiles as olfactory cue, especially ethanol. In order to better understand colonization mechanisms in ambrosia beetles, we carried out an experiment aimed at understanding i) whether colonization success changes when trees are subjected to dummy (ethanol-injection) vs. real (flooding) stress; ii) whether colonization success changes in two common order of trees (Fagales vs Rosales) under the latter different treatments; iii) whether the colonization success changes in different tree species under the different treatments. Eight tree species (four in the orders Fagales and four in the order Rosales) were bought from a local nursery and brought to the natural area of the "Bosco Nordio", where they were subjected to the two treatments and some used as control. Results showed that the injected plants were highly attacked by five common ambrosia beetle species -two exotic and three native- and injected trees were preferred compared to flooded trees, while control trees had no attacks. In relation to the two different orders of tree species and the different tree species, it is interesting to notice that differences in the number of individuals emerged between orders or among species were evident in flooded trees but not on injected trees. This study provides further evidence for the leading role of ethanol in host tissues as a cue for colonization success in ambrosia beetles, but suggesting that the effect of ethanol and the other substances might be species-specific

1. INTRODUCTION

1.1 Ambrosia beetles

The term “ambrosia beetles”, indicates a polyphyletic group of about 5000 insects belonging to the weevil subfamilies Scolytinae and Platypodinae. (Hulcr et al. 2015). Their common name does not refer to a taxonomic group but to an ecological strategy: instead of feeding directly on tree tissues, they are obligately associated with nutritional fungal symbionts (Dzurenko and Hulcr 2022).

Ambrosia beetles are among the smallest weevil species between 2 and 6 mm in length, presenting a cylindrical body, in most cases with a dark brown to black colouration, rounded in front of and rear from a dorsal view with the head hidden under the pronotum (Ranger et al. 2016). Their elongated body is well equipped for boring into wood with cuticular structures that aid in pushing and scraping, such as assorted spines and receptacle denticles on the tibiae (Hulcr et al. 2015). Adult females are generally bigger and able to fly, instead males are flightless, with lighter colours and spheroid (Ranger et al. 2016). Larvae, on the other hand, are apodous, pale and assume the typical C-shape, which is quite common in the entire Coleoptera order (Faccoli et al. 2015).

What distinguishes ambrosia beetles from other bark beetles is the fact that they do not feed on wood but on the symbiotic fungal conidia. These beetles carry fungal spores in an invaginated and membranous structure positioned between the pro and mesothorax called mycangium (Fig.1) (Ranger et al. 2016, Dzurenko and Hulcr 2022). Adult females bore one horizontal tunnel into the xylem, preferably along the trunk or stem transferring the spores of the associated ascomycete fungus, which starts the colonization inside the xylem. From the main tunnel, numerous others tunnels ramify vertically and inside the wood, while some of them are widen into brood chambers where in the distal part eggs will be deposited (Ranger et al. 2016). Larvae move and begin feeding on the fungus within the gallery until they reach the maturity (Hoffman 1941).

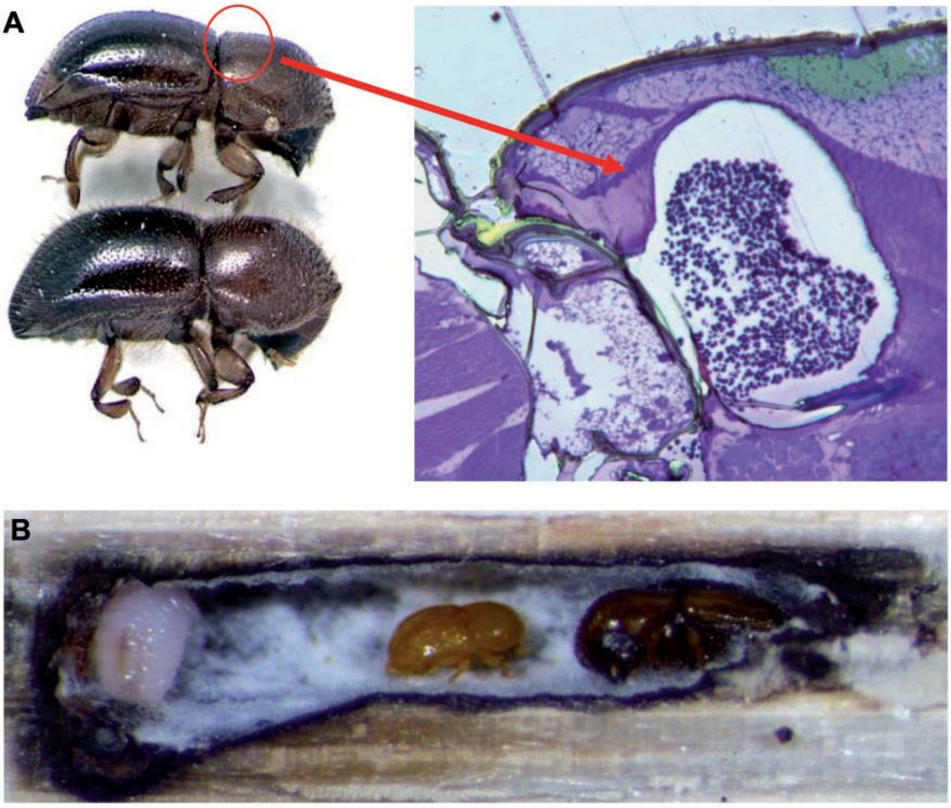

Figure 1. Adult individuals carry their symbiotic fungus within a specialized structure called mycangium. (A) Spores of the symbiotic fungus contained within the mycangium. (B) White mycelium of the symbiotic fungus growing within a gallery containing an immature, adult male, and an adult female (Source: Ranger et al. 2016).

Attacks by ambrosia beetles can result in a rapid dieback and can be difficult to detect due to the small diameter of the gallery entrance (Ranger et al. 2016). In any case, especially for *Xylosandrus* spp., one of the main symptoms is a toothpick-like extrusion of chewed wood in correspondence of the hole, which is the result of the digging activity of the female (Ranger et al. 2016). Damages are also represented by sap flowing out of the entrance hole and tissue discoloration, wilting foliage followed by crown decay and necrosis of branches and trunks (Gugliuzzo et al. 2021).

Among this group of insects, the tribe Xyleborini includes the highest number of exotic species. These insects have an atypical reproductive biology because normally males are haploid (possessing only one set of chromosome) since they arise from unfertilized eggs, while females possess two set of chromosomes (diploid) arising from fertilized eggs (Dzurenko and Hulcr 2022, Ranger et al. 2016). Usually males never leave their maternal galleries and mate with their siblings. Sibling mating combined with haploidy can be one of the reasons for the colonization success of these insects as a single female can establish one population by herself without suffering any inbreeding depression (Dzurenko and Hulcr 2022). For instance, two closely related species, both native to East Asia, the black stem borer (*Xylosandrus germanus*) and the granulate ambrosia beetle (*Xylosandrus crassiusculus*), are major pests in US orchards and tree nurseries (Dzurenko and Hulcr 2022). Thanks to their small size and cryptic habits, ambrosia beetles are easily introduced in non-native area through the trade of wood products (Gugliuzzo et al. 2021). In general, more than 50 species are established outside their native range (Lantschner et al. 2020, Hulcr 2017).

1.2 Host range and mechanisms of host selection in ambrosia beetles

Aggressive ambrosia beetle species in association with their fungal symbionts, such as *Xyleborus glabratus* and certain *Euwallacea* spp., are able to infest and kill healthy trees (Mendel et al. 2021; Chen et al. 2021). These species are known to utilize host volatiles, particularly sesquiterpenoids, for primary location of suitable trees to colonize (Kendra et al. 2014, 2016, 2017). By contrast, a few *Xylosandrus* spp. ambrosia beetles that are associated with branch dieback and tree death in ornamental nurseries and tree fruit orchards preferentially select trees in the early stages of physiological stress (Ranger et al. 2021).

In addition, most of ambrosia beetle is highly polyphagous. For instance, more than 120 host species have been recorded for *X.crassiusculus*, and both *X.compactus* and *X.germanus* were found on more than 200 host species (Greco and Wright 2012, 2015; Ranger et al. 2016; Vannini et al. 2017).

Several ambrosia beetles are attracted by ethanol produced by stressed trees (Cavaletto et al. 2021). The production of ethanol and others volatile compounds (acetaldehyde, acetic acid, ethane, ethylene and methanol) by the plant can be caused by both abiotic and biotic stressors, such as drought, flooding, freezing as well as pathogenic fungi or bacteria (Fig.2) (Ranger et al. 2020). At this regard, several studies using baited-traps (Oliver and Mannion 2001; Miller and Rabaglia 2009; Reding et al. 2011; Galko et al. 2014; Tarno et al. 2021), ethanol-infused or ethanol-injected bolts (Klingeman et al. 2017; Reding and Ranger 2020; Monterrosa et al. 2021), and ethanol-injected or ethanol-irrigated trees (Ranger et al. 2018; Reding et al. 2017; Addesso et al. 2019) highlighted the attractiveness of ethanol for several ambrosia beetle species. For instance, Kelsey et al. (2013) documented that in ethanol-infused sapwood tissue attacks by ambrosia beetle were four times more than in the opposite side of the same log.

Rassati et al. (2020) reported that *Xylosandrus germanus* was less attracted by bolts soaked with high concentration of ethanol while *Xyleborinus saxesenii* showed a positive correlation. Furthermore, from this study emerged also that the presence of the causal agent of the chestnut blight, *C.parasitica*, in chestnut logs can affect host selection in ambrosia beetles as it influences the release of ethanol by the tree. Cavaletto et al. (2021) assessed that some species seek high (90%) or low (5%) ethanol concentration emission, whereas others are less selective and that some insect species have more prominent host preference than others.

1.3 Mechanisms of host-plant colonization in ambrosia beetles

Beside host selection, ethanol benefits ambrosia beetle colonization success but beetles are attracted by different ethanol concentration depending also on the host (Rassati et al. 2016, Reding & Ranger 2020). Ranger et al. (2018) showed that some trees baited with ethanol can be attacked but not colonized by *Xylosandrus germanus* which instead produced offspring in stems of trees irrigated with diluted solutions of ethanol.

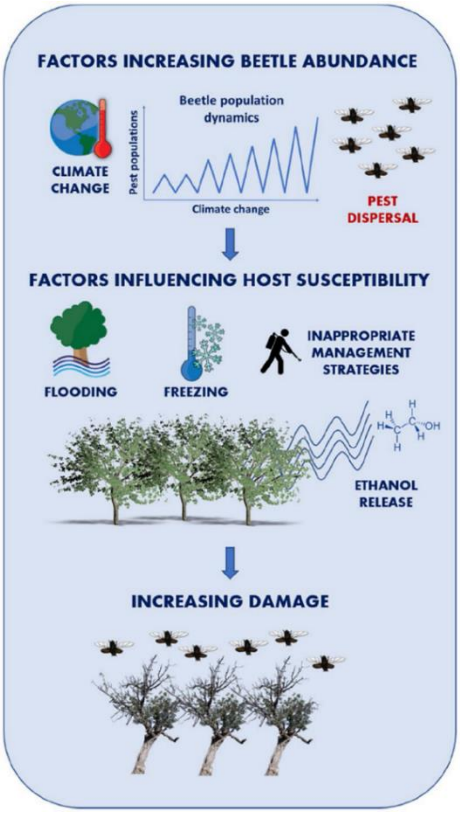

Figure 2. Overview of the main factors increasing ambrosia beetle abundance (Source: Gugliuzzo et al. 2021).

In addition, different tree species emit volatiles that might enhance the competitive ability of mutualistic ambrosia fungi over other fungal antagonists, as ambrosia fungi are able to detoxify ethanol and use it as a carbon source, whereas the antagonists are strongly inhibited in their growth by even small amounts of ethanol, which is typically an antimicrobial compound (McGovern et al. 2004; Tunc et al. 2007).

Moreover, mutualistic ambrosia beetle fungi are known to produce ethanol and other alcohols themselves (Kuhns et al. 2014; Kandasamy et al. 2016), giving them the possibility to enrich the colonized woody substrate with ethanol and thus maintain their dominance even after the production by the dying plant cells ceases (Kimmerer and Kozlowski 1982).

Oviposition does not initiate until the fungal gardens are flourishing and an optimal concentration of ethanol in the tree maximises the growth of symbiotic fungi and inhibits the growth of antagonistic fungi (Lehenberger et al. 2021; Ranger et al. 2018).

From the study of Ranger et al. (2018) emerged that the growth of *Ambrosiella grosmanniae* and *Raffaelea canadensis*, the fungal symbionts of *X.germanus* and *X.saxesenii*, were enhanced when ethanol concentrations in culture media were about 1–2% and 2–3%, respectively. Similarly, ethanol incorporated into agar-based media promoted the growth of nutritional fungal symbionts of *A.dispar*, *X.germanus*, *X.crassiusculus*, and *X.saxesenii*, but inhibited the growth of antagonistic fungi (Lehenberger et al. 2021).

2. OBJECTIVES

The three main objectives of this study are:

(i) to test whether the colonization success of some widely distributed native and exotic ambrosia beetle changes in ethanol-injected vs. flooded trees;

(ii) to test whether the colonization success of the same native and exotic ambrosia beetle changes in two common order of trees (Fagales vs Rosales) under the different treatments;

(iii) to test whether the colonization success of the same native and exotic ambrosia beetle changes in different tree species under the different treatments.

3. MATERIALS AND METHODS

A semi-field experiment was carried out in the Integrated Nature Reserve of the "Bosco Nordio", using eight different species of plants exposed to two different treatments along the control, i.e., direct injection with ethanol inside the plant with the BITE, and flooding, with the root system submerged with water in order to stimulate the natural emission of ethanol from the plant, changing from an aerobic, to anaerobic cellular respiration.

3.1 Bosco Nordio

The study was carried out in Bosco Nordio (Fig.3). Since 1998, Bosco Nordio has been included in the 'Natura 2000 Network' as Site of Community Importance (S.I.C.) and Special Protection Zone (Z.P.S.) no. IT 3250032 pursuant to EEC Directive 92/43 'Habitat' and EEC Directive 79/409 'Birds'.

Bosco Nordio is located in the Veneto region, in the southernmost area of the province of Venice (45.1219444° N; 12.2630555° E;) and it has an extension of about 113 hectares. The area is located in a coastal area where the mouths of three major rivers converge: Po, Adige and Brenta have modelled the territory over the centuries (Rallo 1992).

Bosco Nordio is a broadleaf-dominated forest and represents the remains of old native forests that covered the upper part of the Adriatic coastal area. The forest is dominated by holm oak (*Quercus ilex* L.) and manna ash (*Fraxinus ornus* L.), whereas common oak (*Quercus robur* L.), wild linden (*Tilia cordata* Miller), stone pine (*Pinus pinea* L.), and white poplar (*Populus alba* L.) represented the most common secondary species.

The particular geographic location of Bosco Nordio, together with the diversity of existing biotopes, results in interesting and diversified fauna. In 2000 'Veneto Agricoltura' started the conservation operation are aimed at restoring the area to its natural conditions through floristic re-composition, land restoration and site protection (http://www.parks.it/riserva.bosco.nordio/par.php).

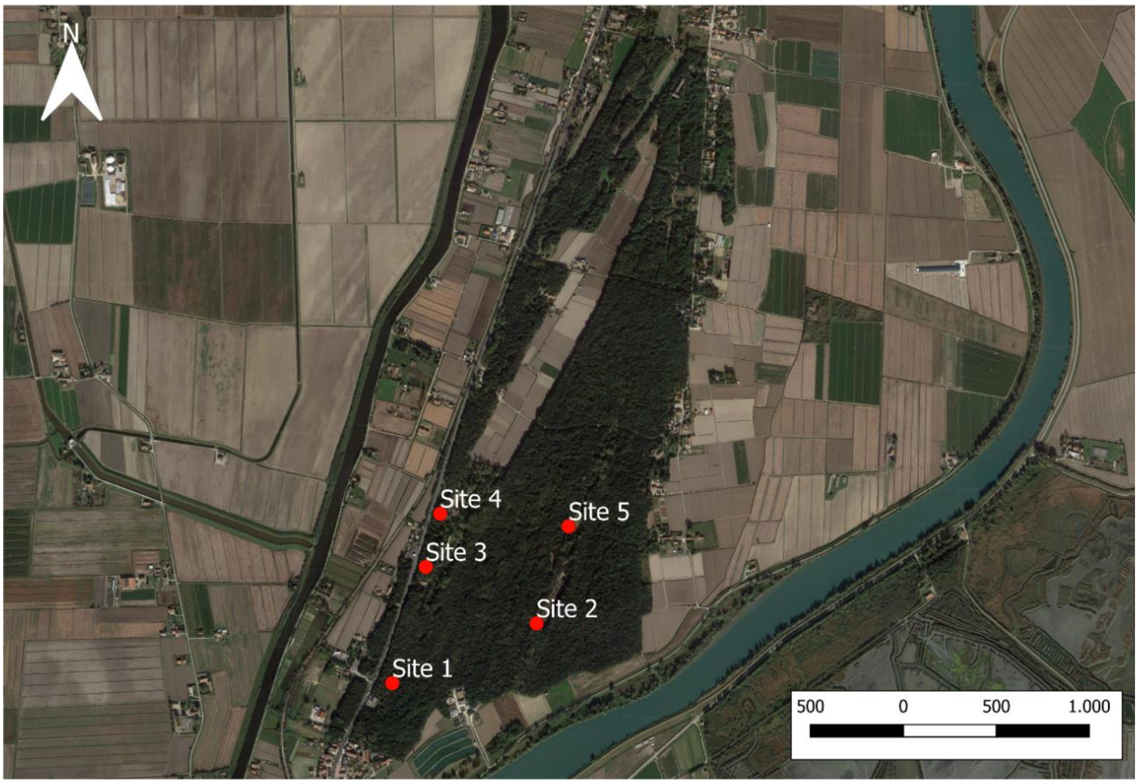

Figure 3. Aerial photo of Bosco Nordio with the localisation of the five sites used for the experiment.

3.2 Tree species

Eight tree species in the orders Fagales and Rosales were bought from a local nursery (Vivai Guagno Ferrara) (Table 1). These tree species were selected because they are commonly used in crop production and forestry and because they are known as potential host of the ambrosia beetle species targeted in this study. The eight tree species were balanced among the orders Fagales and Rosales and were all about the same size (3-5 cm diameter) and age. For each tree species 15 individuals were bought.

Tree species	Order	Family	Common name	Code
Corylus avellana	Fagales	Betulaceae	Hazelnut	**Ca**
Carpinus betulus	Fagales	Betulaceae	European hornbeam	**Cb**
Quercus ilex	Fagales	Fagaceae	Evergreen oak	**Qi**
Quercus robur	Fagales	Fagaceae	Common oak	**Qr**
Malus sylvestris	Rosales	Rosaceae	Apple tree	**Ms**
Prunus armeniaca	Rosales	Rosaceae	Common apricot	**Par**
Prunus avium	Rosales	Rosaceae	Wild cherry	**Pav**
Pyrus pyraster	Rosales	Rosaceae	European wild pear	**Pp**

Table 1. Tree species that were selected for the experiment.

3.3 Design of the experiment and treatments

Individuals of the eight tree species were exposed to two treatments along the control i.e., ethanol-injection and flooding.

Regarding the ethanol-injection, trees were injected with ethanol/water solution (90% ethanol / 10% water) and the amount of the injected solution was adjusted depending on the approximate volume of the tree. The injection was done using the BITE (Fig.4a), a tool developed by the University of Padova (Montecchio 2013), at the base of the trunk. The latter is a manual, drill-free instrument with a small, perforated blade that enters the trunk by separating the woody fibers with minimal friction. The blade reduces the vessels' cross section, increasing sap velocity and allowing the natural uptake of an external liquid to the leaves, when transpiration rate is substantial (Montecchio 2013). The tool has been positioned at around 30 cm from the base of the trunk.

For the flooding treatment (Fig.4c) a pot-in-pot technique was applied. The potted trees have been placed inside another pot filled with water until the soil of the potted tree was supersaturated and plastic bags were used inside the pots to avoid spillage or draining of the water. The permanence of the roots in water should stimulate the production of ethanol in a natural way. Then, the bags were closed using plastic cord. Every week the water level was checked and adjusted if necessary.

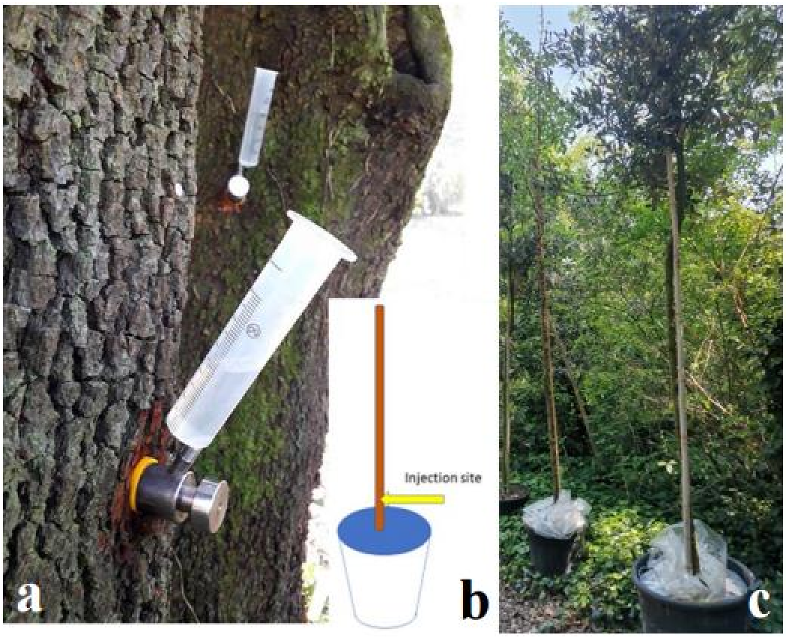

Figure 4. Different treatments used during the experiment: (a) Injection treatment (b) point of injection, (c) flooded plants.

A fully randomized design was used . For each species there were 15 potted trees: 5 injected with ethanol, 5 exposed to flooding stress and 5 left as control units, for a total of 120 trees (Table 2).

For the experiment, the replicas are positioned in four glades inside the Reserve (24 individuals per site), two sites (site 3 and site 4) are located in the same glade. Trees are disposed randomly in a straight line (Fig.5b) at a distance of 2 meters each. To secure the trees metal wires has been used, to avoid them falling during the time of the experiment (Fig.5c).

Figure 5. (a) Glade relative to Site 1, (b) positioning of the trees in a straight line (c) final result after the fastening with metal wires.

Location	Site 1	Site 2	Site 3	Site 4	Site 5
1	Ca	Pp	Ms	Cb	Pav
2	Ca	Par	Ca	Ca	Cb
3	Par	Qi	Cb	Qr	Ca
4	Pp	Pp	Ms	Pp	Pav
5	Ms	Qr	Pp	Pp	Pp
6	Pp	Ms	Pav	Cb	Ca
7	Ms	Ms	Qr	Par	Cb
8	Ms	Par	Cb	Pav	Par
9	Pav	Pav	Qr	Ms	Ca
10	Pav	Ca	Pav	Pp	Ms
11	Par	Qi	Par	Qr	Qr
12	Pp	Pav	Pp	Qi	Qi
13	Cb	Ms	Cb	Ms	Par
14	Qi	Qi	Ms	Par	Ms
15	Cb	Pp	Ca	Par	Cb
16	Qi	Pav	Qr	Pav	Ms
17	Pav	Cb	Par	Qr	Pp
18	Qi	Ca	Qi	Cb	Qr
19	Qr	Ca	Ca	Qi	Qi
20	Par	Qr	Qi	Ca	Qr
21	Qr	Cb	Pp	Qi	Qi
22	Ca	Par	Qi	Ms	Pp
23	Qr	Cb	Par	Pav	Pav
24	Cb	Qr	Pav	Ca	Par

Table 2. Location of the plants in the field. Sites 3 and 4 are located in the same forest glade. Colour key: yellow=control, green=flooded, white= ethanol injection.

The experiment started the 5th of April 2022 and the field activity lasted for six weeks (Table 3). During the first two days all the plant were positioned and settled in different blocks. In the course of the second week, the different treatments were applied and four weeks more passed in order to let the ambrosia beetle colonize the different tree species. All the trees have been controlled every week, watered in order to not let them drying out.

At the end of the sixth week all the treated trees were cut in logs (Fig.6), and brought back to the lab, while the control individuals were left in the field.

Date	Day	Activity
05.04.2022 - 06.04.2022	-	Setting the blocks
19.04.2022	0	First irrigation of the plants
26.04.2022 - 27.04.2022	7	Ethanol injection and flooding started
03.05.2022	14	Sampling week 1
10.05.2022	21	Sampling week 2
17.05.2022	28	Sampling week 3
24.05.2022	35	Sampling week 4

Table 3. Schedule of the monitoring field activity.

Figure 6. The end of the field activities: on the left, the flooded trees ready to be cut, on the right all the cut logs ready to be transported to the laboratory.

3.4 Laboratory activity

Once brought back to the laboratory, the logs were placed in rearing chambers fitted with netting holes (Fig.7) so that air could circulate, preventing damages to the samples. Each chamber was then marked with its species code and stored in the laboratory for five weeks. Every week, all ambrosia beetles emerged from the logs were taken from each box and placed in previously sterilised test tubes.

Figure 7. Rearing chambers conserved in the laboratory divided per species and treatment.

As second step all the emerged ambrosia beetles present in each tube were identified to species and counted. This was done using an electronic stereoscope (Fig.8a) transferring the ambrosia beetles into Petri dishes (Fig.8b).

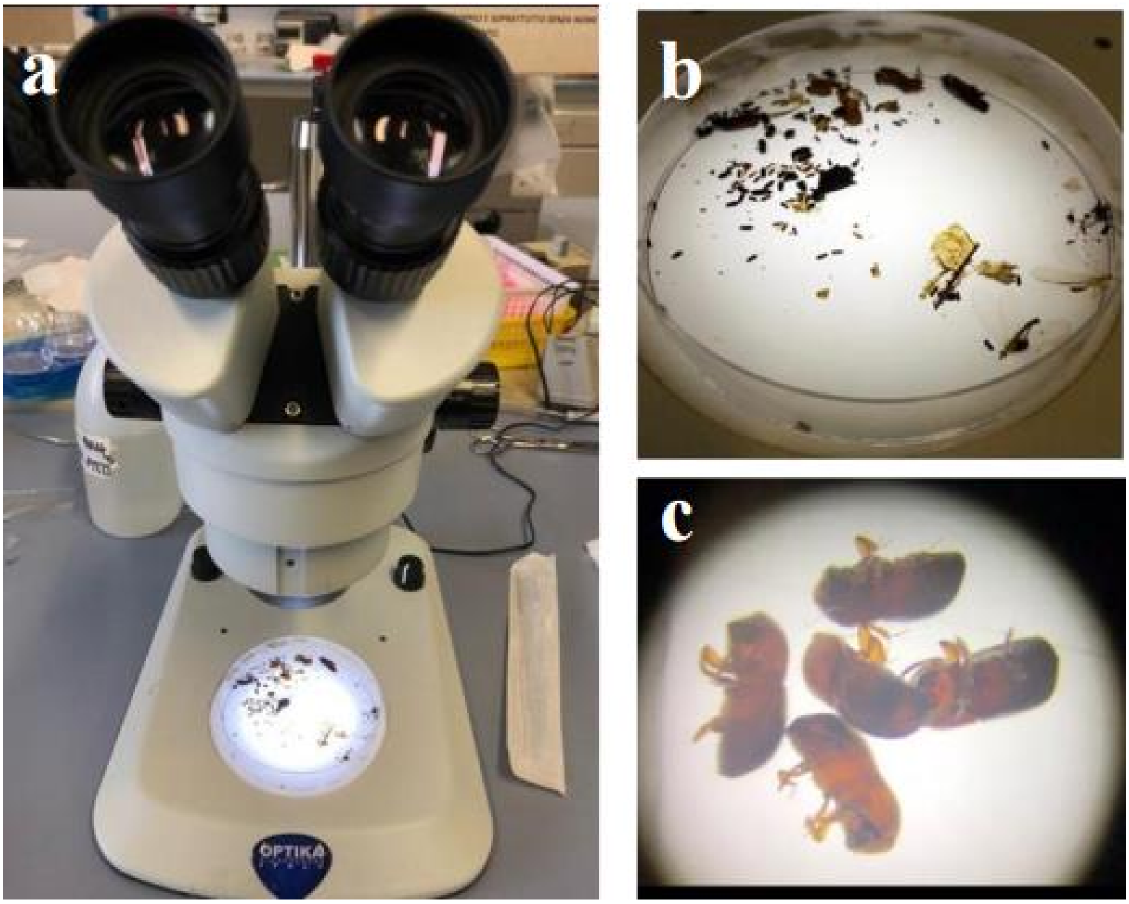

Figure 8. (a) Stereoscope (b) Petri dish with the content of one box (c) ambrosia beetle of the same species.

3.5 Statistical analysis

To evaluate the effect of treatment and the host preference on total beetle emergence, two negative binomial mixed models with a logarithmic link-function were built and validated. The control plants were removed from the models since no beetles emerged from the logs. The response variable was the total number of beetles emerged from the logs. In the first model, the categorical explanatory variables were the plant order (two levels: Fagales and Rosales), the treatment (two levels: flooding and injection), the sampling week (five levels) and the interaction between plant order and treatment. In the second model, the plant order was replaced with the plant species (eight levels).

To evaluate the effect of treatment and the host preference on the emergence of each beetle species, three negative binomial mixed models with a logarithmic link-function were built and validated. The response variables were the number of *Xylosandrus crassiusculus*, *Xylosandrus germanus* and *Xyleborinus saxesenii* emerged from the logs, respectively. *Xyleborus monographus* and *Anisandrus dispar* were not considered in the analyses due to the low number of insects emerged. The categorical explanatory variables were the plant species (eight levels), the treatment (two levels), the sampling week and the interaction between plant species and treatment.

In all models, the plant identity ($n = 80$) was included as a random factor, and the control plants ($n = 40$) were removed since no beetles emerged from the logs. Pairwise multiple comparisons were performed using post-hoc tests with Tukey's correction of p-values (p). Differences were considered significant when $p < 0.05$.

4. RESULTS

4.1 General results

Signs of the presence of ambrosia beetles were already evident just one week after the beginning of the experiment. The first plants to be colonized were the injected trees, irrespective of the tree species. On the contrary the flooded trees started to be attacked during the second week of the experiment while no attacks were detected on control trees.

In general, ambrosia beetle attacks were higher on injected trees than flooded trees. The entrance holes were localized primarily just around the injection site and then spread all along the trunk of the plant (Fig.9a-c).

At the end of the experiment, symptoms on trees (Fig.9b) were more evident on the injected trees, most of which dead. The flooded trees showed weakening and yellowing leaves while control trees showed no symptoms.

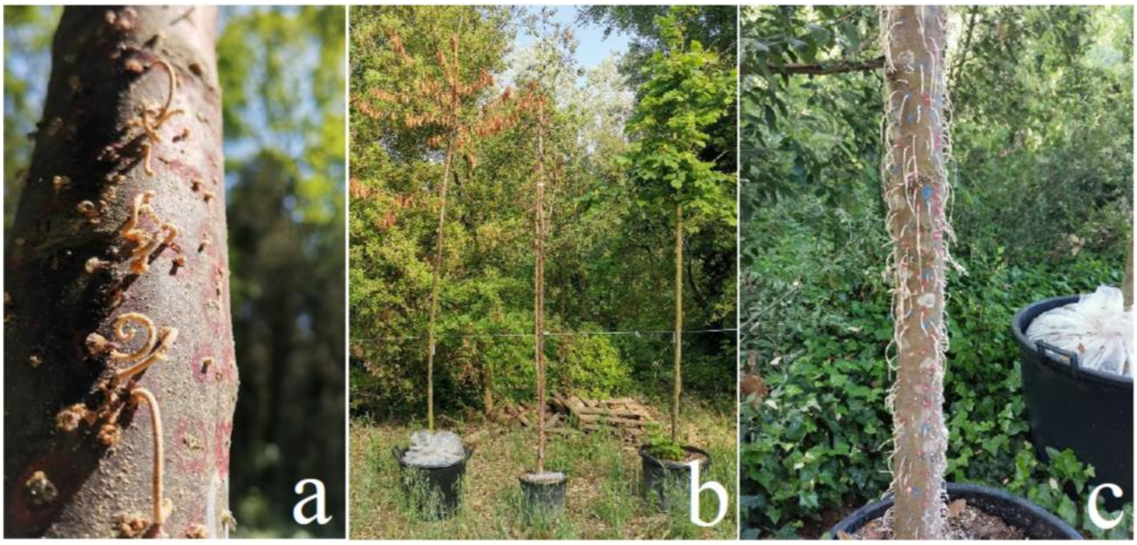

Figure 9. Symptoms of ambrosia beetle presence. (a) toothpick-like extrusion of chewed wood material; (b) trees condition at the end of the experiment; (c) attacks on an injected tree.

4.2 Ambrosia beetle species attacking trees and abundance of emerged individuals

Trees were attacked by five ambrosia beetle species, three native *Xyleborinus saxesenii*, *Xyleborus monographus* and *Anisandrus dispar*, and two exotic *Xylosandrus crassiusculus* and *Xylosandrus germanus*. (Table 4.).

	Week 1	Week 2	Week 3	Week 4	Week 5	**Total**
Xylosandrus crassiusculus	1993	1428	13946	4700	1818	23885
Xylosandrus germanus	70	308	3015	1566	1525	6484
Xyleborinus saxesenii	134	347	699	515	972	2667
Xyleborus monographus	87	159	160	40	18	464
Anisandrus dispar	3	0	0	0	0	3

Table 4. Number of individuals emerged every week in the lab for each of the five ambrosia beetle species.

A total of 33.503 individuals emerged from the logs. The most abundant species was *Xylosandrus crassiusculus* (Fig.10), an exotic species originary from Asia. The total number of individuals counted for this species was 23.885.

Figure 10. Lateral and dorsal view of *Xylosandrus crassiusculus* (Source: https://xyleborini.myspecies.info/taxonomy/term/1168).

The second most abundant species was *Xylosandrus germanus* (Fig.11), originary from Asia and introduced in both Europe and North America. The total number of individuals was 6.484.

Figure 11. Lateral and dorsal view of *Xylosandrus germanus* (Source: https://xyleborini.myspecies.info/taxonomy/term/1176).

The last relevant species in terms of number of emerged individuals was *Xyleborinus saxesenii* (Fig.12), native to Europe but now cosmopolitan. The total number of counted individual was 2.667.

Figure 12. Lateral and dorsal view of *Xyleborinus saxesenii* (Source: https://xyleborini.myspecies.info/taxonomy/term/1219).

The remaining two ambrosia beetle species were the native *X. monographus* and *A. dispar*. *Xyleborus monographus* (Fig.13) typically infests damaged or dying oaks and it has only recently been introduced to the United States. The total number of counted individuals was 464.

Figure 13. Lateral and dorsal view of *Xyleborus monographus* (Source: http://treatment.plazi.org/id/03F2065AF00A2204FF3B9E2EFBB21952).

Anisandrus dispar (Fig.14) is widely distributed throughout Europe, most of Africa, the Middle East and it has also been accidentally introduced in North America. The individuals emerged were however only 3.

Figure 14. Lateral and dorsal view of *Anisandrus dispar* (Source: https://xyleborini.myspecies.info/taxonomy/term/179).

The mean number of emerging individuals per tree was also higher for *X.crassiusculus* (60 ± 9) than *X.germanus* (16 ± 2) followed by *X.saxesenii* (7 ± 0,6), *X.monographus* (2 ± 0.17) and *A.dispar* (0,0075 ± 0,0043) (Fig.15).

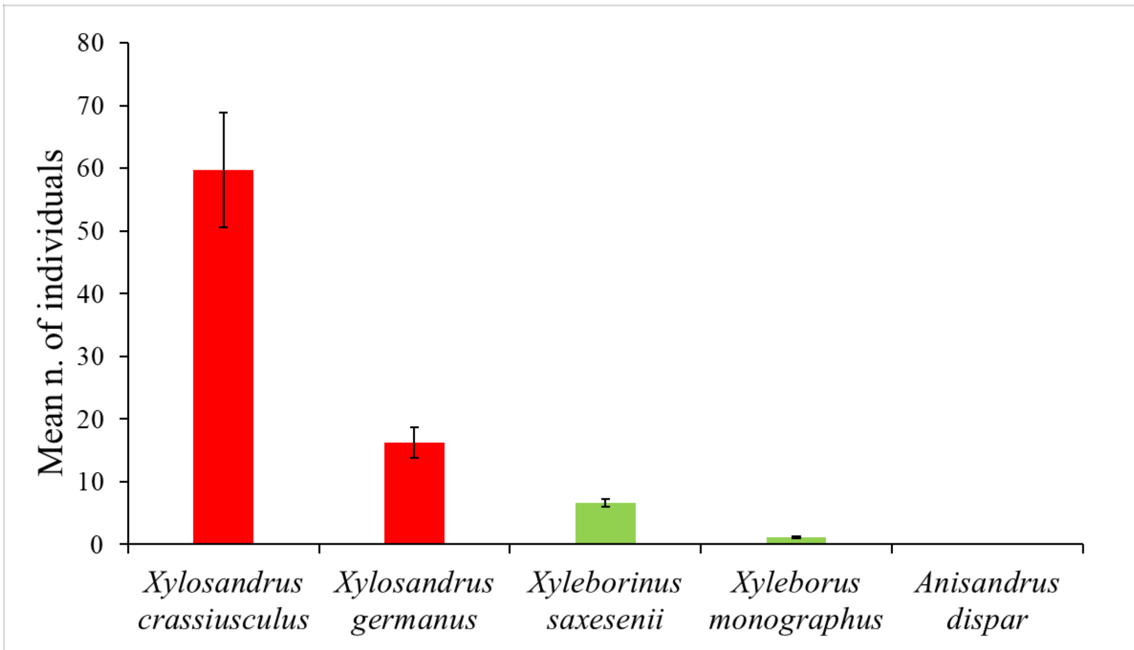

Figure 15. Mean number of each ambrosia beetle species emerged per tree.

4.3 Effect of treatments on ambrosia beetle colonization success

Trees injected with ethanol produced much more individuals than those stressed with flooding (Table 5). In particular, for *X.crassiusculus* 94% of individuals emerged from injected trees while the remaining 6% emerged from flooded trees, while for *X. germanus* 96% individuals emerged from injected trees while from flooded trees emerged the remaining 4%. Regarding *X.saxesenii*, the percentage of individuals emerged from injected trees was 86%, while from the flooded trees only the 14% of individuals emerged. For *X.monographus* 92% of individuals emerged from the injected trees and 8% from the flooded trees. The only three individuals of *A.dispar* emerged from the injected trees.

More in particular, for *X.crassiusculus* a mean number of 112 ± 17 individuals emerged from ethanol-injected trees compared to 7 ± 1 from flooded-trees. For *X.germanus* a mean number of 31 ± 4 from injected trees and a mean number of 1 ± 0.4 for flooded-trees. *X.saxesenii* showed a mean number of 11 ± 1 for the injected trees and 2 ± 0,6 individuals for flooded-trees. For *X.monographus* a mean of 2 ± 0,3 individuals emerged from ethanol-injected trees and 0,7 ± 0,05 individuals from flooded trees, concluding with *A.dispar* with a mean of 0,015 ± 0,0086 individual from injected trees (Fig.16).

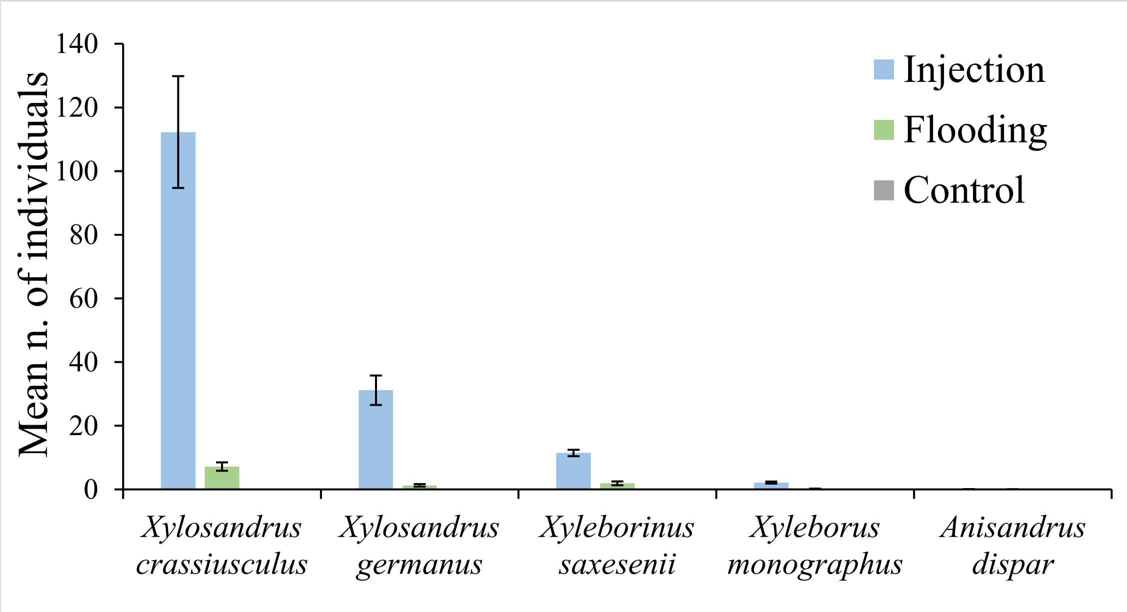

Figure 16. Effect of treatment on total number of individuals emerged per species.

4.4 Effect of tree order on ambrosia beetle colonization success

The interaction between tree order and treatment had a significant effect on the total number of individuals emerged from the trees (p<0.001) (Fig.17). In particular, the mean number of individuals was higher for Rosales than Fagales in flooded trees but did not differ among the two tree orders when trees were injected with ethanol.

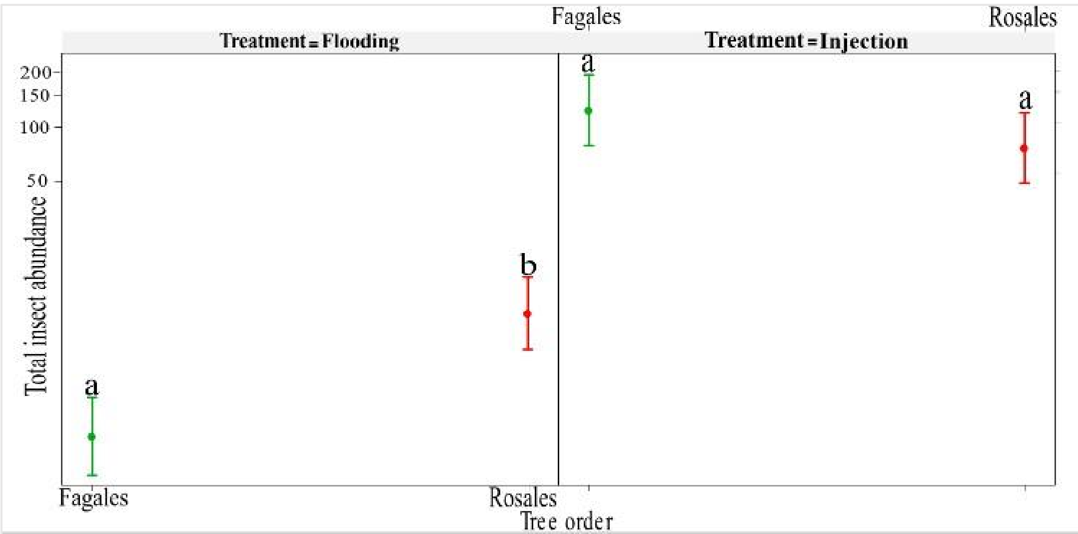

Figure 17. Comparison of ambrosia beetle individuals emerged from Fagales vs Rosales under the two treatments (ethanol-injection vs flooding). The red bars correspond to the Rosales order, while the green bars represent the Fagales order.

4.5 Effect of tree species and treatments on ambrosia beetles colonization success

The interaction between tree species and treatment had a significant effect on the total number of individuals emerged from the trees ($p<0.001$) (Fig.18). Specifically, the mean number of individuals was significantly higher for *Malus sylvestris* (Ms), *Prunus armeniaca* (Par), *Prunus avium* (Pav) and *Quercus ilex* (Qi) than *Corylus avellana* (Ca) *Carpinus betulus* (CB), and *Quercus robur* (Qr) in flooded trees but no difference emerged when trees were injected with ethanol.

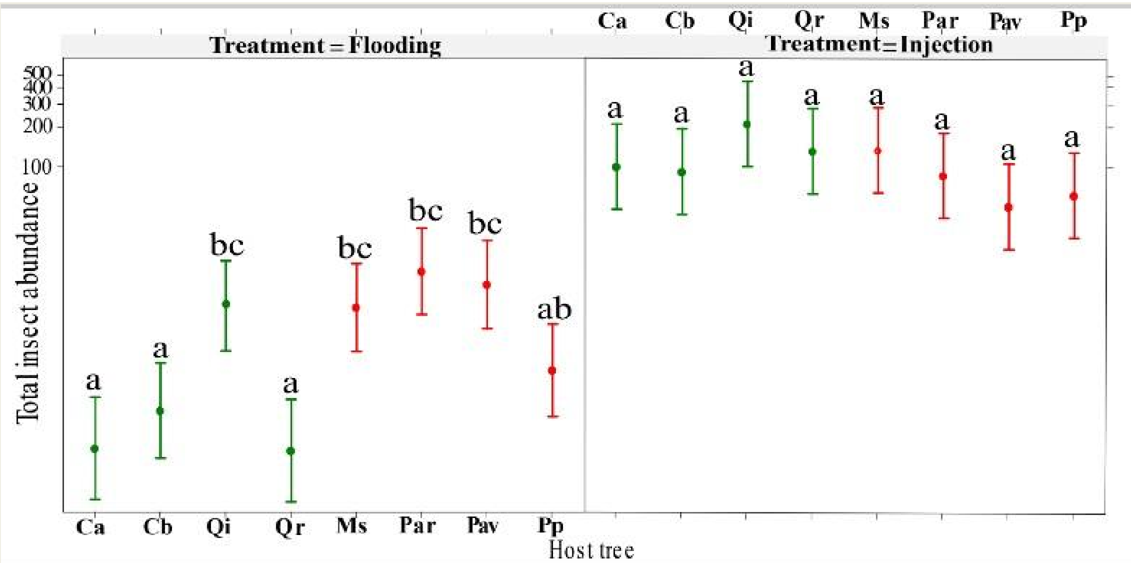

Figure 18. Comparison of ambrosia beetle individuals emerged from the 8 tree species under the two treatments (ethanol-injection vs flooding). The red bars correspond to the Rosales order, while the green bars represent the Fagales order.

4.5.1 Xylosandrus crassiusculus

For *X.crassiusculus*, the interaction between tree species and treatment had a significant effect on the total number of individuals emerged from the trees ($p<0.001$) (Fig.19). In particular, the mean number of individuals was significantly higher for *Quercus ilex* (Qi) than *Prunus avium* (Pav) and *Pyrus pyraster* (Pp) in injected trees but for the flooded trees the mean number of individuals was higher not only for *Quercus ilex* (Qi) but also *Prunus armeniaca* (Par), compared to *Quercus robur* (Qr), *Corylus avellana* (Ca) and *Carpinus betulus* (Cb).

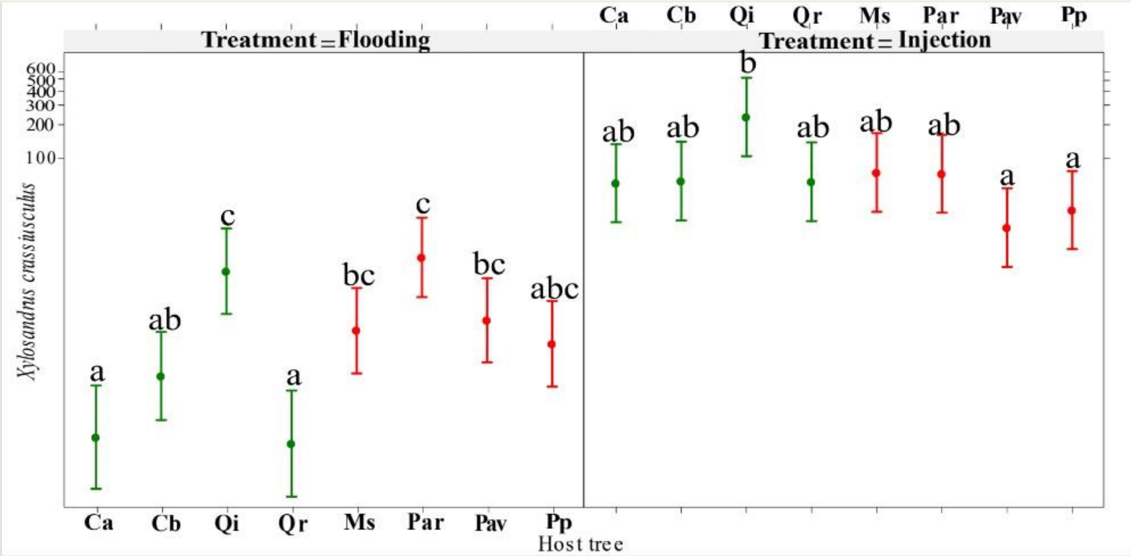

Figure 19. Comparison of *Xylosandrus crassiusculus* individuals emerged from the 8 tree species under the two treatments (ethanol-injection vs flooding). The red bars correspond to the Rosales order, while the green bars represent the Fagales order.

4.5.2 Xylosandrus germanus

For *X.germanus*, the interaction between tree species and treatment had a significant effect on the total number of individuals emerged from the trees ($p<0.001$) (Fig.20). Specifically, the mean number of individuals was significantly higher for *Quercus robur* (Qr) than *Prunus armeniaca* (Par) for injected trees, but the trend was different for flooded trees i.e., the mean number of individuals was higher for *Malus sylvestris* (Ms) than *Corylus avellana* (Ca), *Carpinus betulus* (Cb) *Quercus robur* (Qr) and *Pyrus pyraster* (Pp).

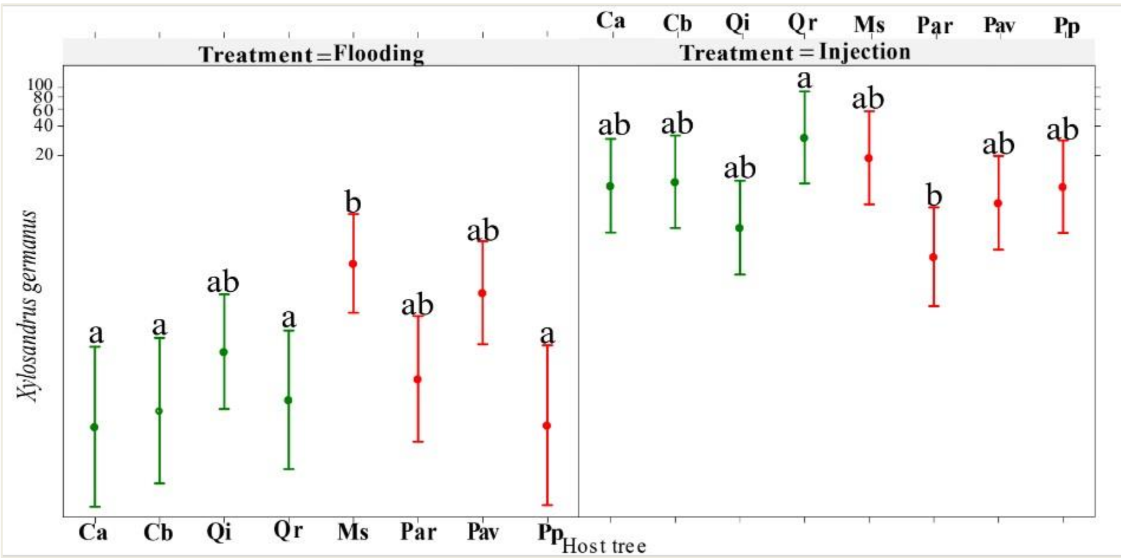

Figure 20. Comparison of *Xylosandrus germanus* individuals emerged from the 8 tree species under the two treatments (ethanol-injection vs flooding). The red bars correspond to the Rosales order, while the green bars represent the Fagales order.

4.5.3 Xyleborinus saxesenii

For *X.saxesenii*, the interaction between tree species and treatment had a significant effect on the total number of individuals emerged from the trees ($p<0.001$) (Fig.21). In particular, in flooded trees the mean number of individuals was significantly higher for *Prunus avium* (Pav) and *Prunus armeniaca* (Par), than *Carpinus betulus* (Cb), *Quercus robur* (Qr) and *Pyrus pyraster* (Pp) but no difference emerged when trees were injected with ethanol.

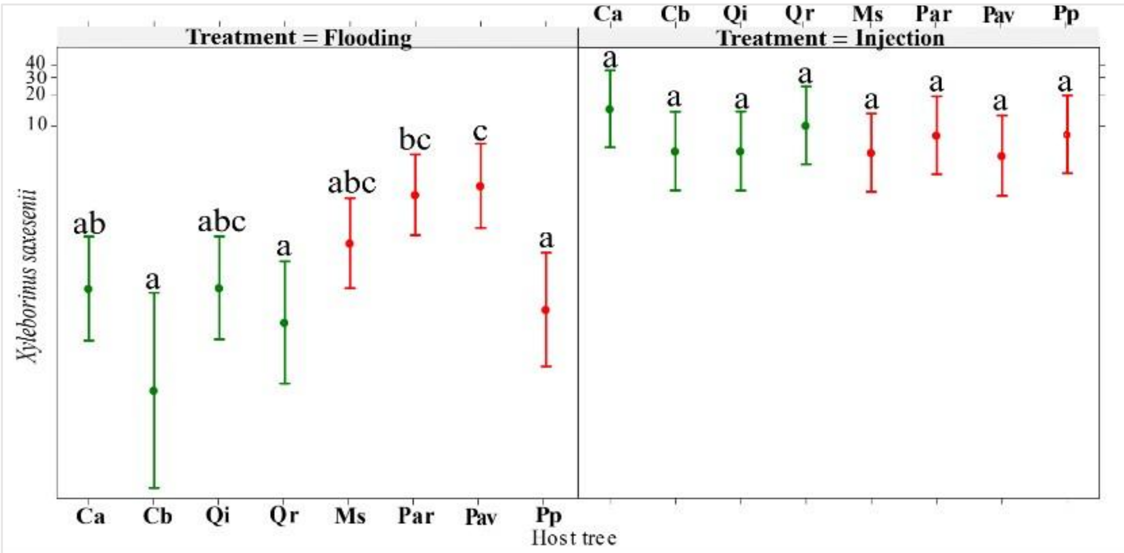

Figure 21. Comparison of *Xyleborinus saxesenii* individuals emerged from the 8 tree species under the two treatments (ethanol-injection vs flooding). The red bars correspond to the Rosales order, while the green bars represent the Fagales order.

5. DISCUSSION

Nowadays, the study of the ambrosia symbiosis is among the most active directions in insect symbiosis research globally (Hulcr and Stelinski 2017). Their broad host range, the nutritional symbiosis with fungi, the inbreeding-mating, the efficiency at locating and colonizing trees under physiological stress render beetle management challenging and often ineffective (Gugliuzzo et al. 2021). Some ambrosia beetles are the main responsible for damage in ornamental plant nurseries and orchards, and understanding factors that affect host selection and colonization is necessary to improve management strategies for both native and exotic species.

Ethanol released by the tree, aids several species of ambrosia beetles in locating suitable hosts to colonize and can enhance the growth of their fungal nutritional symbionts (Ranger et al. 2018), nevertheless is still unclear whether different ambrosia beetle species show preference for certain ethanol concentration over others.

In this study 120 trees equally divided between species of the order Fagales (4 species) and Rosales (4 species) were set in “Bosco Nordio” for four weeks with the aim to find out whether the effect of host species on ambrosia beetle colonization success changes depending on the type of stress applied to them, comparing a real stress i.e. flooding, vs. a dummy stress i.e. ethanol-injection.

From the analysis emerged that the most abundant species are exotic ambrosia beetles species, *X.crassiusculus* and *X.germanus*, which are extremely successful invaders able to cause severe damages, to attack a broad host range, and to easily detect ethanol produced by the tree even in low concentration. This is in line with what was found by Cavaletto et al. (2021) for *X.crassiusculus*, which attack number was high even in logs filled with a low ethanol-concentration and by Ranger et al. (2021) for *X.germanus*, reporting that the latter species preferentially attacks stressed trees thanks to an efficient olfactory mechanism able to orient towards host-derived volatile cues, especially ethanol.

The other native ambrosia beetle species found in this study, *Xyleborinus saxesenii, Xyleborus monographus* and *Anisandrus dispar* are all known to be attracted by ethanol-emitting trees.

Results showed that all treated trees have been attacked, but that the injected trees were preferred in respect to the flooded trees while control trees had no attacks.

The absence of attacks on control trees by ambrosia beetles further demonstrate a preference for (and ability to) efficiently locate physiologically-stressed hosts, using ethanol concentration as an indicator of host tree susceptibility.

The clear preference for ethanol-injected vs. flooded trees might be explained by the fact that a very high amount of ethanol was injected and that the other volatiles produced by stressed trees might not be important as ethanol for colonization success. In flooded trees, other volatiles (such as acetaldehyde, methanol, and acetone) were likely produced but they likely had a negligible effect for the colonization success of the ambrosia beetles considered in this study. This is confirmed by previous study comparing the attractiveness of ethanol and other stress-induced and host-derived compounds for ambrosia beetles, showing that most of these compounds have generally a negligible effect (Montgomery and Wargo 1983). In particular, the mixture of acetaldehyde, ethanol, and methanol was not more attractive to scolytines than ethanol alone. Similarly, Ranger et al. (2010) assessed a similar pattern for *X. germanus*, showing that ethanol was highly attractive, methanol was slightly attractive, and acetaldehyde and acetone were inactive. Nonetheless, it must be pointed that the amount of ethanol and the other volatiles in the different tree species under the two treatments has not been measured yet, and this info will be essential to better understand the observed patterns.

In relation to the two different orders of tree species, it is interesting to notice that there are not evident differences in the number of individuals emerged from tree species belonging to the Rosales order and the Fagales order when trees were injected with ethanol, while significant differences emerged when comparing the two orders in flooded trees. A very similar trend was observed when comparing the number of individuals emerged from the tested tree species under the two treatments. The high amount of ethanol in the injected trees may cover the species-specific differences that may be among the different tree species, as confirmed in the study of Reding et al. (2016), where in ethanol-injected trees attacks were more strongly influenced by ethanol-injection than tree species.

Among the flooded individuals, the observed differences can be explained by the fact that certain species, and in general those belonging to the Rosales order, are likely to be more susceptible to flooding, producing the amount of ethanol required by ambrosia beetles to successfully reproduce inside the wood. This is in accordance with what is reported by Ranger et al. (2015b) where *X.germanus* and *X.crassiusculus* distinguished among tree species varying in their tolerance of flood-stress and preferentially selected intolerant over tolerant species.

A very similar pattern, with differences among individuals emerged from the different tree species more evident in flooded than ethanol-injected trees, was found when analysing the different ambrosia beetle species singly. Nonetheless, the species-specific response was different in the different ambrosia beetle species, showing specific preferences.

For *X.crassiusculus* the comparison of individuals emerged from the 8 tree species under the flooding treatment shows a preference to the species belonging to the Rosales order, while for *X.germanus* the difference between the flooded species is less evident. This is an interesting result that need further development because it may mean that this response could be due to the fact that different plant species subjected to a real stress such as flooding, probably develop amounts of ethanol and other substances that create ideal conditions for some ambrosia beetle species but not for others.

Lastly, as regard *X.saxesenii*, the comparison of individuals emerged from the 8 tree species under the two treatments (ethanol-injection vs flooding) shows less difference both among treatments and species, suggesting an ability to attack trees with different ethanol concentration and under various stages of decline. This result is in line with other studies as the study of Cavaletto et al. (2021) in which the preference of different concentration of ethanol has been tested for different ambrosia beetle species and it emerged that *X. saxesenii* attacked bolts infused with 5% or 90%.

6. CONCLUSION

Abiotic stressors such as flooding induce a tree to produce ethanol and consequently triggering ambrosia beetle attacks in ornamental plant nurseries, orchards and natural settings. This study provides further evidence for the leading role of ethanol in host tissues as a cue for colonization success in ambrosia beetles, but suggesting that the effect of ethanol and the other substances might be species-specific.

Nevertheless, information on the actual content of ethanol and other volatiles in host tissues is required to understand mechanisms behind the observed patterns.

Additional studies are required to improve the understanding of the colonization patterns of ambrosia beetles together with the role of stress-related volatiles in ambrosia beetle, information that can strongly improve our ability to predict the most endangered tree species and improve management strategies.

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