



Species-specific effects of ethanol concentration on host colonization by four common species of ambrosia beetles

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Abstract

Ambrosia beetles (Coleoptera; Curculionidae; Scolytinae and Platypodinae) can cause severe damage to trees growing in plant nurseries, orchards and natural forests. Ethanol is emitted by stressed trees and represents an important cue used by ambrosia beetles to locate suitable hosts to infest. Ethanol also favors the growth of ambrosia beetles' nutritional fungal symbionts and suppresses the growth of antagonistic fungi. An optimal concentration of ethanol in host tissues might maximize fungal growth and offspring production, but it is unclear if this optimal concentration varies among ambrosia beetle species. To investigate this mechanism, we injected five different concentrations of aqueous ethanol solution (5%, 25%, 50%, 75% and 90%) into the stems of container-grown oak trees, *Quercus robur* L. Modified Falcon tube chambers were used to confine four species of field-collected ambrosia beetles to the injected stems, namely, *Anisandrus dispar*, *Xyleborinus saxesenii*, *Xylosandrus germanus*, and *Xylosandrus crassiusculus*. Incidence of boring, ejected sawdust, gallery development, and offspring production were then quantified. The incidence of boring generally increased with increasing ethanol concentration for all four Scolytinae species tested. Ejected sawdust and offspring production increased with increasing ethanol concentration up to 90% for *A. dispar* and *X. saxesenii*; by contrast, an increasing trend up to 75% ethanol followed by a decrease at 90% ethanol was associated with *X. germanus* and *X. crassiusculus*. Our study highlights the key role of ethanol for ambrosia beetles, and showed that the optimal concentration maximizing colonization and offspring production can vary among species.

Keywords *Anisandrus dispar* · Colonization mechanisms · *Xyleborinus saxesenii* · *Xylosandrus crassiusculus* · *Xylosandrus germanus*

Key message

- Ambrosia beetles can cause damage to a wide range of host trees
- Ethanol aids several species of ambrosia beetles during host selection and colonization
- Preference of different ambrosia beetle species for specific ethanol concentrations is unclear
- Ethanol concentration injected into potted trees differentially affected colonization by ambrosia beetles
- Optimal ethanol concentration maximizing colonization and offspring production varies among species

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Introduction

Fungus-farming ambrosia beetles (Coleoptera: Curculionidae) represent about 3400 species within the Scolytinae and 1400 species within the Platypodinae (Hulcr et al. 2015). These beetles, and especially those in the tribe Xyleborini, are recognized as extremely successful invaders worldwide (Hulcr and Stelinski 2017). More than 50 species are already established outside their native range (Lantschner et al. 2020), and other species are expected to arrive in the near future due to ever increasing international trade and accessibility to new species pools following diversification of trade partners (Brockerhoff and Liebhold 2017). In addition, climate change is predicted to favor ambrosia beetle introductions in areas where their establishment is currently impeded by climatic constraints (Urvois et al. 2021; Pureswaran et al. 2022). Despite most ambrosia beetle invasions going unnoticed due to the lack of visible economic or ecological impacts, some species have become serious pests of trees growing in plant nurseries, orchards, urban areas and natural settings (Ranger et al. 2016; Hughes et al. 2017; Ploetz et al. 2017; Gugliuzzo et al. 2021). Thus, understanding factors affecting host selection and colonization success is considered a research priority to aid in developing management strategies.

Aggressive ambrosia beetle species in association with their fungal symbionts, such as *Xyleborus glabratus* Eichhoff 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). Ethanol is emitted by trees in response to a variety of stressors and represents an important cue for both *Xylosandrus* spp. and other ambrosia beetle species for locating suitable hosts to infest (Ranger et al. 2015a,b, 2021; Wheeler et al. 2021; Dzurenko and Hulcr 2022). The attractiveness of ethanol to ambrosia beetles was demonstrated in several studies using ethanol-baited traps (Oliver and Mannion 2001; Miller and Rabaglia 2009; Reding et al. 2011, 2013a; 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. 2010, 2012, 2018; Reding et al. 2013b, 2017; Adesso et al. 2019). The release rate of ethanol from baited-traps and the concentration of ethanol injected into trees also influence ambrosia

beetle attraction (Klimetzek et al. 1986; Kelsey and Joseph 1997, 1999; Ranger et al. 2012). For instance, the number of entry holes by *Xylosandrus germanus* (Blandford) into ethanol-soaked bolts was negatively correlated with increasing ethanol concentration while a positive correlation was documented for *Xyleborinus saxesenii* Ratzeburg (Rassati et al. 2020). Similarly, bolts filled with 5% or 90% ethanol were differentially attacked by *Anisandrus dispar* (Fabricius), *Xylosandrus crassiusculus* (Motschulsky) and *X. saxesenii*, whereby *X. crassiusculus* preferentially bored into bolts infused with 5% ethanol, *A. dispar* preferentially selected bolts infused with 90% ethanol, and *X. saxesenii* equally selected bolts irrespective of ethanol concentration (Cavaletto et al. 2021).

Besides host selection, ethanol influences ambrosia beetle host colonization by promoting the growth of their nutritional symbiotic fungi. For example, *X. germanus* established fungal gardens and produced offspring in stems of container-grown trees irrigated with dilute ethanol solutions while no fungal gardens or offspring were recorded from superficial galleries created in stems of ethanol-baited trees in which tissues did not contain ethanol (Ranger et al. 2018). 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 (Ranger et al. 2018; Lehenberger et al. 2021). Growth of *Ambrosiella grossmanniae* and *Raffaella 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 (Ranger et al. 2018). These results suggest an optimal concentration of ethanol might exist within host trees according to interspecific variability in the tolerance of ambrosia beetle nutritional fungal symbionts to ethanol (Ranger et al. 2018; Lehenberger et al. 2021).

Based on the aforementioned studies, we hypothesized that ethanol concentration within host trees influences ambrosia beetle boring, gallery development, and offspring production, and that interspecific variability exists among ambrosia beetles for different concentrations of in vivo ethanol. To test this hypothesis, we used trunk injections of varying ethanol concentrations to assess interspecific variability on boring activity and colonization patterns of four ambrosia beetles that are widespread worldwide: *A. dispar*, *X. saxesenii*, *X. germanus* and *X. crassiusculus*.

Materials and methods

Study site and ethanol injection

The experiment was conducted in 2021 at the Agripolis campus of the University of Padova, Legnaro, Italy (45° 20'

39° N; 11° 57' 15" E). Forty potted common oaks (*Quercus robur* L.) were obtained from a tree nursery located in Ferrara, Emilia Romagna, Italy (44° 39' 52" N; 12° 7' 38" E) and were brought to the experimental site at the beginning of March. The latter was represented by a greenhouse tunnel with a roof covered with a clear plastic film and a shading net (Fig. S1A). The common oak was selected as it is a potential host species for all four target species (Maksymov 1987; Cavaletto et al. 2021). All trees were containerized in 35 L containers, and were between 3.4 and 5.4 cm in diameter (mean = 4.27 ± 0.08 cm) (Table S1). The tree height was approximately 3.5 m. Potted trees were randomly assigned to five aqueous ethanol solutions (v/v) containing 5%, 25%, 50%, 75%, 90% ethanol and were then set up in a complete randomized design ($n = 8$ trees per ethanol concentration) (Fig. S1A). Lowest and highest ethanol concentrations were selected based on previous host selection and colonization studies (Klingeman et al. 2017; Ranger et al. 2018; Cavaletto et al. 2021). We did not include control trees (i.e., trees not injected with ethanol) as a previous study showed that adult females of *X. germanus* and *X. crassiusculus* do not start boring activity when confined with no-choice to stems of trees that do not contain ethanol (Ranger et al. 2015b).

Ethanol solutions were injected using a tool (BITE) developed by the University of Padova (Montecchio 2013). 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 (Fig. S1B). 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). Injection site was always located at 30 cm from the tree base. Previous studies involving injected trees used 75 ml of ethanol solution for 4.5 cm diameter tree (Ranger et al. 2010; Reding et al. 2013b); this value was used as baseline for this experiment, and reduced or increased depending on the tree volume (Table S1). Ethanol solution was injected every two weeks for about three months (12 May, 26 May, 9 June, 23 June, 7 July, 21 July, 9 August) using a syringe inserted in the BITE system (Fig. S1B). This time interval was selected as the ethanol concentration in tissues of injected trees was found to strongly decrease after two weeks (Ranger et al. 2012). Over the interval between two ethanol-injections, we continuously injected water into the tree using a catheter bag connected to the BITE system in order to avoid wound closure and the entry of air into tree vessels (Montecchio 2013) (Fig. S1C). All potted trees were irrigated twice per day during the duration of the experiment.

Caging of beetles and colonization

Sixteen chambers consisting of 50 ml Falcon tubes were set up along the stem starting from 30 cm above the

injection site and keeping 10 cm between adjacent tubes (Figs. S1D and S2A). The bottom of the Falcon tube cap was cut off and the remaining part was tied with iron wire to the tree stem and then sealed with silicone (Fig. S1D). Chambers were randomly assigned to the four target species within the four sections in which the tree stem was divided (Fig. S2A) in order to have four adult beetles per each ambrosia beetle species per tree. To favor the entry of adult females in the tree stem, we initially reduced the room within each chamber by using a metal disk (Fig. S1D). A total of 640 chambers were set up, 160 for each target ambrosia beetle species. Ambrosia beetles were caged after the first two injections.

Hand-made panel traps (Fig. S3) baited with ethanol were used to collect living individuals of *A. dispar*, *X. crassiusculus*, *X. germanus*, and *X. saxesenii*. Ethanol was released at a rate of 15.8 g/day from 20 holes drilled with a 6.35 mm drill bit on a 50 ml Falcon tube located in the middle of the upper part of the trap (Fig. S3). The trap collector cup was filled with crumpled moist paper towels in which ambrosia beetles can bore and survive for some days. Forty traps were spread across several broadleaved-dominated forest patches located in the Eugean hills area, Veneto region, where all target species are known to occur (Cavaletto et al. 2020). All traps were hung approximately 1 m from the ground, a height at which ambrosia beetles are generally abundant (Marchioro et al. 2020; Miller et al. 2020). Traps were activated at the end of April and checked weekly targeting the activity peak of the target species. On the check date when a species was abundant, all traps were emptied and immediately reactivated; after two days, living individuals were collected, brought back to the lab, identified to species, and divided in groups of five. The individuals within each of the latter groups were then randomly assigned to five potted trees, one per each ethanol concentration. Only individuals that were apparently active and healthy were retained for the experiment. This procedure was repeated until all 160 individuals needed to fill the chambers assigned to each species were caught.

One week after the initial caging, we checked all chambers and recorded where boring activity occurred. The chambers were monitored once per month for the following three months, and ejected sawdust (Fig. S2B, C) and emerged individuals (Fig. S2D) were removed. Ejected sawdust was collected in mini paper bags, dried at 50 °C for a week, and then weighed. Emerged individuals were sexed and counted. At the end of October, tree stem sections associated with each chamber were dissected using a chisel to determine the presence/absence of eggs, larvae, pupae, and adults within each gallery. In addition, galleries were classed as i) superficial; ii) developed without offspring; or iii) developed with offspring (Fig. S4).

Statistical analysis

The effect of ethanol concentration and ambrosia beetle species on the number of entry holes was tested with a linear model using the “*lm*” function implemented in R (R Core Team 2021). The model included the total number of entry holes per tree as the dependent variable, and ethanol concentration (categorical variable), ambrosia beetle species (categorical variable) and their interaction were independent variables. The same approach was used to test the effect of ethanol concentration on the number of entry holes and number of galleries of a certain class for each ambrosia beetle species. For the entry holes, the model included the total number of entry holes per tree as the dependent variable, and ethanol concentration as the independent variable. For galleries, the model included the total number of galleries of a certain class per tree (either developed without offspring or developed with offspring) as the dependent variable, and ethanol concentration as the independent variable. When necessary, the number of entry holes or the number of galleries was log-transformed to satisfy the assumption of normality. The post hoc Tukey’s HSD test (“*emmeans*” function from R “*emmeans*” package) was used to perform pairwise comparison of the treatment groups and test for significant differences between their means.

The effect of ethanol concentration on the amount of ejected sawdust and the number of offspring produced by caged females for each ambrosia beetle species was tested using the nonparametric Kruskal–Wallis test (H statistic) with correction on multiple comparisons according to the Benjamini–Hochberg procedure because the data were not normal and the variance was not homogenous. The model included either the amount of ejected sawdust in grams or the total number of offspring per caged female (calculated pooling together larvae, pupae, and adults but always excluding the foundress) as dependent variables and ethanol concentration as the independent variable. Analyses were carried out with the function “*kruskal.test*” available for R (R Core Team 2021). The pairwise Wilcoxon test was used to perform pairwise comparison of the treatment groups and test for significant differences between their means

(function “*pairwise.wilcox.test*” package “*stats*”). The same approach described above was used to test the effect of the distance (in cm) from the injection site on the amount of ejected sawdust and offspring for each ambrosia beetle species.

For all analyses, alpha was set at 0.1 a priori to also account for marginally significant differences given the exploratory nature of our study and the intrinsic variability expected to exist among potted trees.

Results

Boring activity and colonization pattern differed among ambrosia beetle species. *Xylosandrus crassiusculus* and *A. dispar* showed the highest incidence of boring (i.e., percentage of foundresses that started boring) (47.5% and 46.8%, respectively), followed by *X. germanus* and *X. saxesenii* (24.37% and 18.75%, respectively) (Table 1). *Xylosandrus crassiusculus* established the highest number of developed galleries containing offspring (Table 1), while most of the galleries created by *A. dispar* were either superficial or did not contain offspring (Table 1). *Xylosandrus crassiusculus* was also the species producing the highest number of both females and males, followed by *X. saxesenii*, *X. germanus* and *A. dispar* (Table 1).

Amount of ejected sawdust and offspring were not affected by the distance from the injection site in any of the ambrosia beetle species tested (Table S2).

Effect of ethanol concentration on boring activity

The number of entry holes on potted trees was affected by both ethanol concentration and ambrosia beetle species ($F_{19,140}=7.63$, $p < 0.001$), with no interaction between the two variables. The number of entry holes was significantly higher on trees injected with 75% and 90% ethanol than trees injected with 5%, 25% and 50% ethanol (Fig. 1A), as well as on trees injected with 50% ethanol than in trees injected with 5% and 25% ethanol. In addition, the number of entry holes

Table 1 Total number of galleries recorded per each class considered in this study and total number of larvae, pupae and adults (not counting the foundress) obtained for each beetle species. The latter values represent the sum of offspring emerged and found within the gallery

	Abundance of attack outcomes				Abundance of offspring stages			
	Failed	Superficial	Developed without offspring	Developed with offspring	Larvae	Pupae	Females	Males
<i>Anisandrus dispar</i>	85	30	34	11	1	–	55	22
<i>Xyleborinus saxesenii</i>	130	6	8	13	23	–	252	13
<i>Xylosandrus crassiusculus</i>	84	20	16	40	–	–	728	44
<i>Xylosandrus germanus</i>	121	11	14	14	–	3	152	16

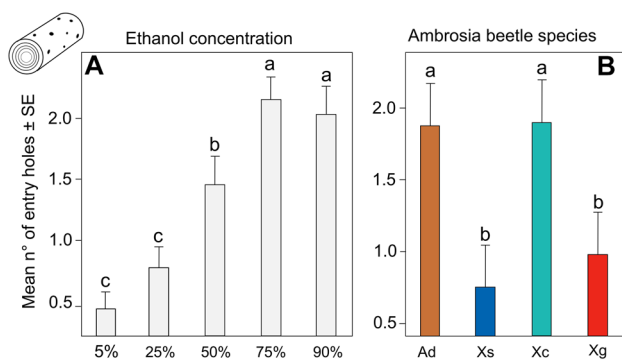


Fig. 1 Effect of ethanol concentration (A) and ambrosia beetle species (B) on total number of entry holes per potted tree. Ad=*Anisandrus dispar*; Xs=*Xyleborinus saxesenii*; Xc=*Xylosandrus crassiusculus*; Xg=*Xylosandrus germanus*. Means with the same letter are not significantly different using Tukey's HSD test

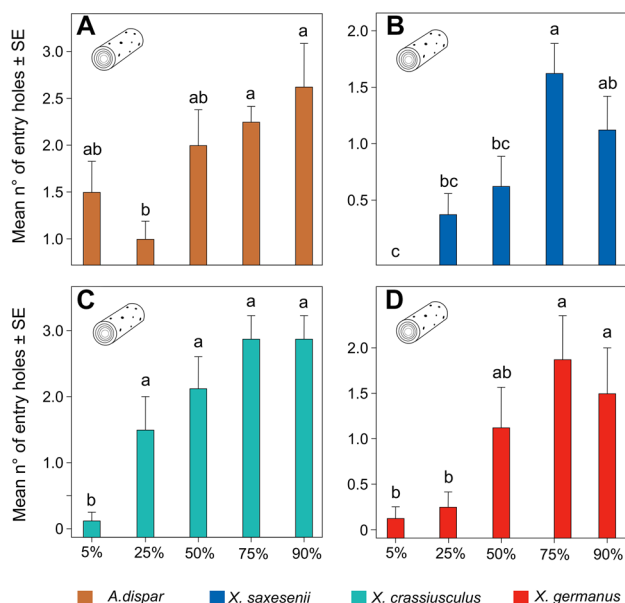


Fig. 2 Effect of ethanol concentration on the number of entry holes per potted tree by *A. dispar* (A), *X. saxesenii* (B), *X. crassiusculus* (C), and *X. germanus* (D). Means with the same letter are not significantly different using Tukey's HSD test

was significantly higher for *A. dispar* and *X. crassiusculus* than both *X. saxesenii* and *X. germanus* (Fig. 1B).

At the species level, ethanol concentration affected the number of entry holes in all ambrosia beetle species ($F_{4,35} = 3.30$, $p = 0.02$ for *A. dispar*; $F_{4,35} = 8.60$, $p < 0.001$ for *X. saxesenii*; $F_{4,35} = 13.47$, $p < 0.001$ for *X. crassiusculus*; $F_{4,35} = 4.98$, $p = 0.002$ for *X. germanus*). For *A. dispar*, the mean number of entry holes per tree was significantly higher in trees injected with 75% and 90% ethanol than trees injected with 25% ethanol (Fig. 2A), but no significant difference was observed among trees injected with

5%, 50%, 75%, and 90% ethanol. For *X. saxesenii*, the mean number of entry holes per tree was significantly higher in trees injected with 75% ethanol than trees injected with 5%, 25% and 50% ethanol (Fig. 2B), whereas no difference was observed with trees injected with 90% ethanol (Fig. 2B). For *X. crassiusculus*, the mean number of entry holes on trees injected with 5% was significantly lower than trees injected with the other tested concentrations (Fig. 2C). For *X. germanus*, the mean number of entry holes per tree was significantly higher on trees injected with 75% or 90% ethanol than in trees injected with 5% or 25% ethanol (Fig. 2D).

Effect of ethanol concentration on gallery types

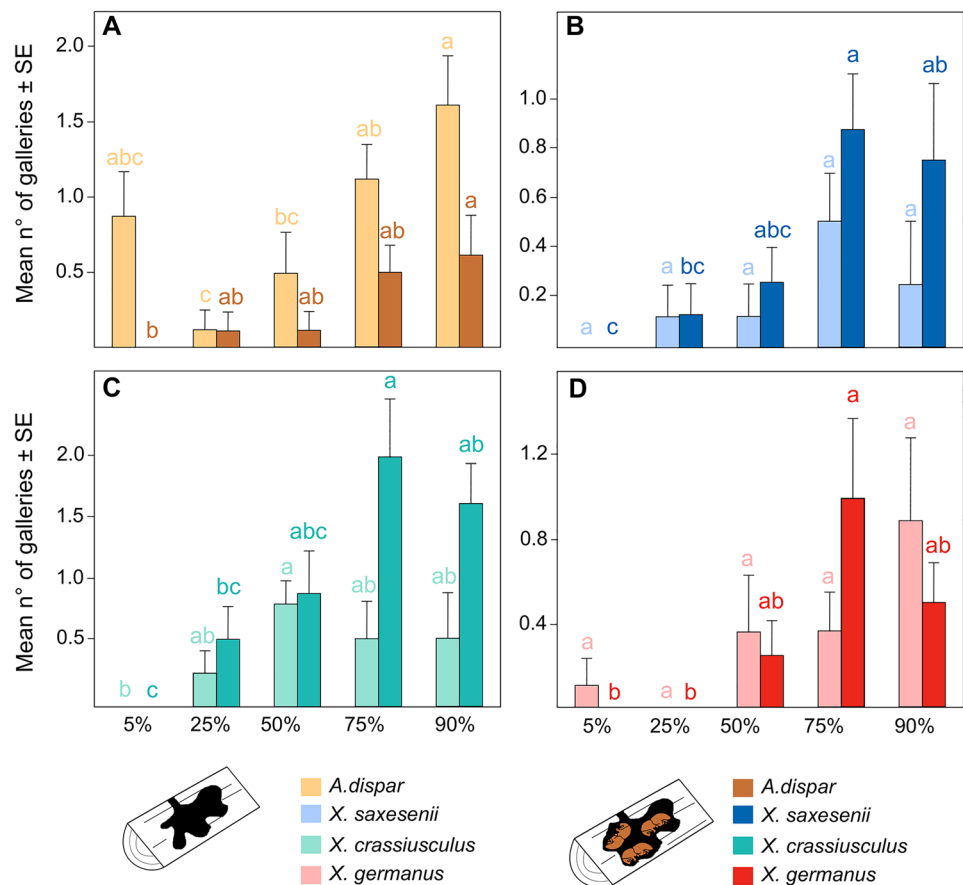
For *A. dispar*, both the number of galleries developed without offspring and galleries developed with offspring were significantly affected by ethanol concentration ($F_{4,35} = 5.11$, $p < 0.002$ and $F_{4,35} = 2.70$, $p = 0.04$, respectively). The trend was different for the two gallery types: the number of galleries without offspring was significantly higher in trees injected with 90% ethanol than in trees injected with 25% and 50% ethanol but did not differ from trees injected with the other tested concentrations (Fig. 3A). The number of galleries with offspring was significantly higher in trees injected with 90% than in trees injected with 5% but did not differ from the other tested concentrations (Fig. 3A).

For *X. saxesenii*, ethanol concentration significantly affected the number of galleries with offspring ($F_{4,35} = 4.35$, $p = 0.005$) but not the number of galleries without offspring ($F_{4,35} = 1.91$, $p = 0.12$). The number of galleries with offspring was significantly higher in trees injected with 75% ethanol than trees injected with 5% and 25% ethanol but did not differ from trees injected with 50% and 90% ethanol (Fig. 3B).

For *X. crassiusculus*, ethanol concentration significantly affected both the number of galleries without offspring ($F_{4,35} = 5.94$, $p < 0.001$) and with offspring ($F_{4,35} = 2.70$, $p = 0.04$). The trend was different for the two gallery types: the number of galleries with offspring was significantly higher in trees injected with 75% ethanol than in trees injected with 5% and 25% ethanol but did not differ from trees injected with 50% and 90% ethanol (Fig. 3C); for the number of galleries without offspring a significant difference was found only between trees injected with 50% and trees injected with 5% ethanol (Fig. 3C).

For *X. germanus*, ethanol concentration significantly affected the number of galleries with offspring ($F_{4,35} = 4.50$, $p = 0.004$) but not the number of galleries without offspring ($F_{4,35} = 1.83$, $p = 0.14$). The number of galleries with offspring was significantly higher in trees injected with 75% ethanol than trees injected with 5% and 25% ethanol but

Fig. 3 Effect of ethanol concentration on the number of galleries bored with and without offspring per potted tree for *A. dispar* (A), *X. saxesenii* (B), *X. crassiusculus* (C), and *X. germanus* (D). Means with the same letter are not significantly different using Tukey's HSD test



did not differ from trees injected with 50% and 90% ethanol (Fig. 3D).

Effect of ethanol concentration on ejected sawdust and offspring

For *A. dispar*, the effect of ethanol was more evident on ejected sawdust ($H = 22.07$, $p < 0.001$) than on offspring production ($H = 12.55$, $p = 0.01$). The mean amount of ejected sawdust was higher on trees injected with 75% ethanol than on trees injected with 5%, 25% or 50% ethanol (Fig. 4A), whereas a difference in the number of offspring was observed only between trees injected with the highest (i.e., 90%) and lowest ethanol concentrations (i.e., 5% and 25%) (Fig. 4B).

For *X. saxesenii*, ethanol similarly affected both sawdust ($H = 14.22$, $p < 0.006$) and offspring ($H = 18.52$, $p < 0.001$) production. Both values were significantly higher in trees injected with 90% ethanol than in trees injected with 5%, 25%, and 50% ethanol (Fig. 4C, D).

For *X. crassiusculus*, ethanol significantly affected both the amount of ejected sawdust ($H = 34.72$, $p < 0.001$) and the number of offspring ($H = 27.52$, $p < 0.001$). In both

cases, significantly higher values were observed in trees injected with 75% and 90% ethanol than in trees injected with 5% and 25% ethanol (Fig. 4E, F).

Ethanol significantly affected both the amount of ejected sawdust ($H = 18.73$, $p < 0.001$) and the number of offspring ($H = 21.15$, $p < 0.001$) also in *X. germanus*. The mean amount of ejected sawdust was higher in galleries excavated on trees injected with 75% ethanol than on trees injected with 5%, 25% and 50% but did not differ from trees injected with 90% ethanol (Fig. 4G). The mean number of offspring recorded in trees injected with 75% ethanol was higher than trees injected with the other tested concentrations (Fig. 4H).

Discussion

Understanding factors that affect host selection and colonization in ambrosia beetles is necessary to improve management strategies for *Xylosandrus* spp. and the other non-native species introduced outside their native range (Ranger et al. 2016; Gugliuzzo et al. 2021). Ethanol aids several species of ambrosia beetles in locating suitable hosts to colonize and can enhance the growth of their nutritional fungal symbionts

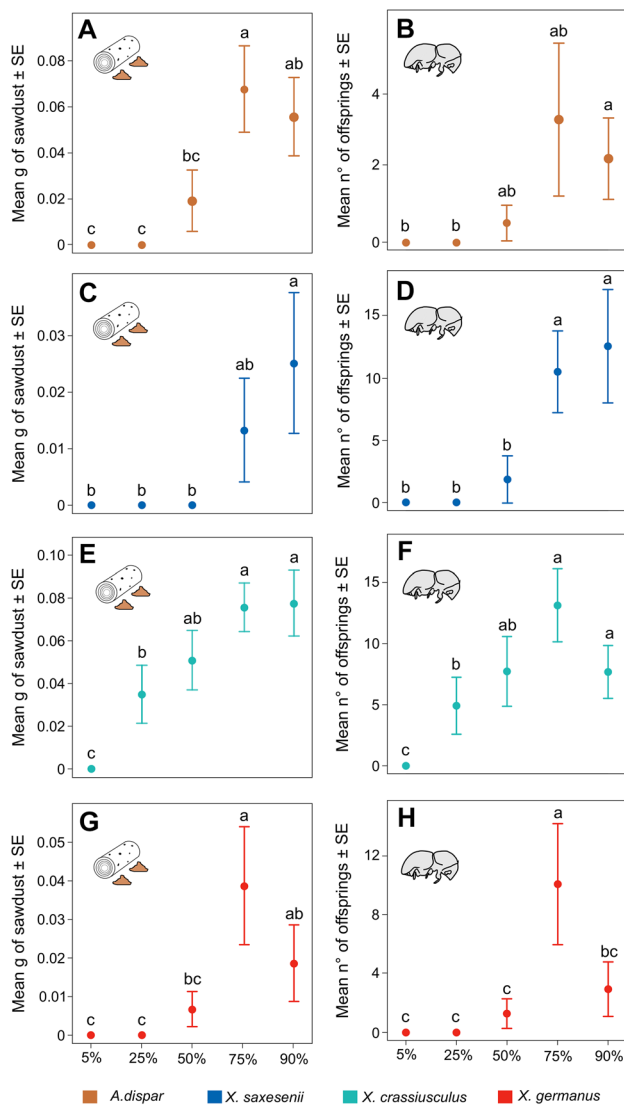


Fig. 4 Effect of ethanol concentration on the mean amount of sawdust and offspring produced per caged female in *A. dispar* (A, B), *X. saxesenii* (C, D), *X. crassiusculus* (E, F), and *X. germanus* (G, H). Means with the same letter are not significantly different using the pairwise Wilcoxon test

(Ranger et al. 2015a,b, 2018). Nonetheless, whether different ambrosia beetle species show preferences for certain ethanol concentrations over others is still unclear. We showed that varying the concentration of ethanol injected into potted trees differentially affected boring activity, gallery development and colonization success in different ambrosia beetle species, thereby supporting the role of in vivo ethanol concentration in determining their ecological niche.

We found that the boring activity on potted trees was generally affected by the amount of ethanol injected, whereby a higher number of entry holes was recorded on trees containing higher ethanol concentrations. Our results confirmed that ambrosia beetles are able to perceive differences in ethanol

concentrations emitted and contained by host trees, and that they preferentially bore into trees containing certain ethanol concentrations over others (Ranger et al. 2012, 2015b). We also found that *A. dispar* and *X. crassiusculus* were generally more likely than *X. germanus* and *X. saxesenii* to initiate boring into injected trees. For *A. dispar* and *X. crassiusculus* ethanol might be the main olfactory cue triggering boring activity (Cavaletto et al. 2021), while *X. saxesenii* and *X. germanus* might require a more complex blend including ethanol and other host related volatiles (Yang et al. 2018; Chen et al. 2021).

At the species level, we found a species-specific effect of ethanol concentrations on the number of entry holes. A differential attractiveness of traps, bolts or trees emitting different amounts of ethanol to different ambrosia beetle species has been demonstrated in previous studies (Klimetzek et al. 1986; Ranger et al. 2011; Cavaletto et al. 2021), even though the response was often inconsistent. In our study both *X. saxesenii* and *X. germanus* preferentially started boring in trees injected with high ethanol concentrations, whereas *A. dispar* and *X. crassiusculus* were less selective and started boring also in trees injected with low ethanol concentrations. These results generally align with previous studies showing *X. saxesenii* to be attracted by traps, trees or bolts associated with high ethanol release rates or high ethanol concentrations (Klimetzek et al. 1986; Rassati et al. 2020) and *X. crassiusculus* being able to bore also on trees with low amount of ethanol (Cavaletto et al. 2021), but are inconsistent with some of the previous findings for *X. germanus*, for which the number of entry holes in ethanol-soaked bolts was found to decrease with increasing ethanol content (Rassati et al. 2020), and for *A. dispar*, which started boring in bolts filled with 90% ethanol but not in bolts filled with 5% ethanol (Cavaletto et al. 2021). The lack of significant difference among attacks registered on trees injected with the highest and the lowest ethanol concentration for *A. dispar* was particularly unexpected given that the attraction of this species to ethanol was shown to increase with increasing ethanol concentration (Klimetzek et al. 1986; Schroeder and Lindelöw 1989). The intrinsic variability existing among potted trees and the possible effect of not noticeable biotic stressors (e.g., fungi or bacteria) in one or a few trees among those injected with 5% ethanol might explain this result. In any case, the comparison among studies based on different methodological approach is complicated as most studies, including ours, did not provide information on the amount of ethanol actually present in host tissues.

We also found that ethanol concentration significantly affected boring activity with a mixed response among species. In general, galleries producing offspring were more abundant in trees injected with one of the two highest ethanol concentrations than with 5% and/or 25% ethanol for all species, but the peak was reached at 90% ethanol for *A.*

dispar and at 75% ethanol for *X. saxesenii*, *X. crassiusculus*, and *X. germanus*. These results confirmed that ethanol concentration in wood tissues is a key parameter determining whether an ambrosia beetle species can successfully reproduce on a given host (Ranger et al. 2018) and that different species can show preferences for certain ethanol concentrations (Rassati et al. 2020; Cavaletto et al. 2021). Testing a higher number of concentrations in the range 50–90% is however required to further investigate species-specific preference patterns and to unravel the optimal ethanol concentration for each species. Besides galleries with offspring, all species bored galleries that did not produce offspring, which number was affected by ethanol concentration in *X. crassiusculus* and *A. dispar* but not in *X. germanus* and *X. saxesenii*. Boring activities in non-reproductive hosts was already observed in previous studies (Rassati et al. 2016, 2020; Cavaletto et al. 2021; Mendel et al. 2021), highlighting that ethanol can act as signal promoting boring activities but also that other factors determine the suitability of the host tissue as reproductive substrate.

Ethanol concentration affected the amount of sawdust ejected by adult females during boring activities and offspring production in all tested species, but the trend varied among species. *Xylosandrus germanus* showed an increase and then a decrease in offspring (but not sawdust) production with increasing ethanol concentration; for *A. dispar*, *X. saxesenii*, and *X. crassiusculus* the amount of sawdust and offspring generally increased with increasing ethanol concentration, although for *X. crassiusculus* a slight but not significant decrease was observed at 90% ethanol. In addition, *X. crassiusculus* was the only species able to produce offspring also in trees injected with low ethanol concentration (i.e., 25%). Offspring production is dependent on the growth of the nutritional fungal symbionts on gallery walls, which was shown to be enhanced by ethanol content and maximized at certain ethanol concentrations (Ranger et al. 2018; Lehenberger et al. 2021). *Ambrosiella grosmaniae*, the main nutritional fungal symbionts of *X. germanus*, was shown to grow better at lower ethanol concentration than *Raffaelea canadensis*, one of two main nutritional fungal symbionts of *X. saxesenii* (Ranger et al. 2018), which in turn benefits of ethanol presence less than *Ambrosiella hartigii*, the main nutritional fungal symbionts of *A. dispar* (Lehenberger et al. 2021). These patterns might explain the decrease of offspring production observed for *X. germanus* in trees injected with 90% ethanol compared to trees injected with 75% ethanol, and the general good performance of both *X. saxesenii* and *A. dispar* also in trees injected with 75% and 90% ethanol. *Ambrosiella roeperi*, the main nutritional fungal symbiont of *X. crassiusculus*, was instead shown to have a lower ethanol tolerance than *A. grosmaniae* (Ranger et al. 2018), but this trend is apparently in contrast with our results. Nonetheless, it might be possible that the optimal

ethanol concentrations maximizing offspring production in *X. crassiusculus* can be observed at a concentration between 50 and 75% that we did not test in this study.

Abiotic stressors such as flooding or poor drainage causing a tree to produce ethanol and consequently triggering ambrosia beetle attacks are common in ornamental plant nurseries, orchards and natural settings (Ranger et al. 2016, 2021; Agnello et al. 2017; Gugliuzzo et al. 2019). Our study provides further evidence for the key role of ethanol concentration in host tissues as a driver of host selection and colonization success in ambrosia beetles, and highlights that the optimal concentration can vary among species. Further studies are however required to improve our understanding on the mechanisms and variables involved. First of all, we tested five different ethanol concentrations (5%, 25%, 50%, 75%, 90%) covering a wide gradient, but our results suggest that the optimal concentrations for the different species might be between the tested concentrations. Unraveling the optimal concentration for the most aggressive ambrosia beetle species might aid in developing species-specific management protocols based on ethanol-baited traps (Ranger et al. 2021) or ethanol-injected trap trees (Addesso et al. 2019). In addition, we did not measure the amount of ethanol actually present in host tissues, a parameter that is basic to compare studies using different methodological approaches. Furthermore, injecting ethanol solutions into healthy trees can be limiting, as a real biotic or abiotic stressor (e.g., flooding, freezing) can cause other volatiles and substances (e.g., monoterpenes, acetic acid, acetaldehyde) to be produced in host tissues in higher quantities, which might be required by some ambrosia beetle species to enhance colonization success. Finally, using a higher number of replicates on a larger spatial scale and testing other tree species along with oak would allow to overcome limitations related to the relatively low number of replicates that we used in this study and the possible effect of the specific physiology and xylem characteristics of the tested oak species.

Author contributions

DR, GC, CMR, MER and LM conceived the study; DR and GC wrote the manuscript; GC conducted field experiments; DR and GC analyzed the data; DR provided funds. All authors contributed to and approved the manuscript.

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Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or vertebrates performed by any of the authors.

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