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Vitex agnus-castus cannot be used as trap plant for the vector Hyalesthes obsoletus to prevent infections by 'Candidatus Phytoplasma solani' in northern Italian vineyards: experimental evidence

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The authors thank the Editor and the anonymous Reviewers for their valuable comments and their time. Following each comment, you´ll find authors response (**Answer**).

Comments of Editor:

Title. I agree with Reviewer 1 in that the title could be substantially improved and altered from a question to a definitive statement.
Answer. We modified the title in accordance with the suggestion by the reviewer (see lines 1-3 of the new version of the manuscript).

Approach. Please carefully consider the comments of Reviewer 2 which has suggested that further information on the phytosanitary status of the plants used in the experiments be included to determine whether these may have affected the volatiles. Additional information is requested on the instrumentation used in the experiment, and total numbers of insects analyzed in the field experiments.
Answer. We inserted the requested details (see lines 142 and 153-155 of the new version of the manuscript), we improved the olfactometer description and the phytosanitary status of the plants (see lines 169-171 of the new version of the manuscript)

Methods. Please pay particular attention to include additional information necessary in the methods to outline the robustness of the approach. These include: the method for collection of H. obsoletus adults, how many were in each cage, etc.
Answer. We are grateful to the editor and reviewers for these comments allowing the improvement of the manuscript We improved the requested data through the all manuscript

Manuscript Preparation. Please check the entire manuscript for typographical errors noted by both reviewers.
Answer. Done. See the improvement through the manuscript in the "with_truck_change" file

Comments of Reviewers:

Reviewer 1:

This is a very interesting study on the possible role of Vitex agnus-castus in BN epidemiology in North Italy, either as attractant plant for vector control strategy or as a common host plant of vector and the pathogen with its own role in the epidemiology. It is a carefully written and well designed study. It gives important new information on the host plant association of H. obsoletus and its specialization towards specific natural host plants. Very important are details on the ability of vector originating from one natural host plant to transmit the pathogen onto other host plant. This study opens a new area of study on the role of V. agnus-castus as constituent in the epidemiological cycle of BN in the coastal zone of Italy.

Manuscript is well written, results are clearly presented and findings are mostly well discussed. I have only minor suggestions for improvement of the clarity of experimental design and suggestions regarding some aspects of discussion.

Title:

Please consider modifying the title in context that it gives an answer to raised question. This is just a suggestion. It could be something like: "Vitex agnus-castus cannot be used as trap plant for the vector Hyalesthes obsoletus to prevent infections by 'Candidatus Phytoplasma solani' in Northern Italy vineyards: experimental evidence"

Answer. We modified the title in accordance with the suggestion by the reviewer (see lines 1-3 of the new version of the manuscript).

Abstract:

- Lines 32 and 35: Please either change "Israel", or change "Eastern Europe". Since you are comparing two geographic regions where Vitex agnus-castus is a host plant of H. obsoletus, please use comparable geographic qualifier. Meaning, use both "Israel" and "Montenegro", or use "east Mediterranean coast of Israel" and "east Adriatic coast of Montenegro". East Europe is too wide geographical term, while it is important to point out that this plant is only growing in the coastal area.

Answer. We modified the wording in accordance with the suggestion by the reviewer. (see lines 32 and 36 of the new version of the manuscript).

- Lines 43-45: I think you should be careful about statement under point (iv) because of the characterization of the transmitted genotype. It is true that H. obsoletus originating from nettle transmitted 'Ca. P. solani' to chaste tree, but it remains questionable did this genotype originated from nettle. I give more details about these findings in the discussion section, but I would suggest to changing this sentence into: "(iv) H. obsoletus originating from nettle is able to transmit 'Ca. P. solani' to chaste tree (two plants out of 16 were found infected by the BN phytoplasma strain St5 identified in H. obsoletus specimens)"

Answer: We modified the sentence at point (iv) in accordance with the reviewer (see lines 44-45 of the new version of the manuscript), and we improved the discussion based on the indication given here below

Introduction:

- Line 91: Replace "recent" with "most recent"

Answer: Done (see lines 97 of the new version of the manuscript).

- Lines 86-94: I understand the storyline, which is gradually leading to Vitex, but tuf-b BN epidemiological cycle sourced by Vitex is evidenced in the east Mediterranean coast of Montenegro (Kosovac et al., 2016). It seems to me that this should be presented alongside with other proven epidemiological cycles of BN.

Answer: We modified the introduction in accordance with the reviewer (see lines 99-100 of the new version of the manuscript).

- Line 121: Please replace "Eastern Europe" with more precise geographic qualifier, such as "east Adriatic coast of Montenegro".

Answer: We modified the wording in accordance with the reviewer throughout the manuscript (see new version of the manuscript).

Material and Methods:

- Lines 138-144: Please describe the method used for collection of H. obsoletus adults. Please give details on the number of insects confined on each plant, source of each plant (grown from seeds, or tissue culture, or taken from field as small plant and then potted) and size of each plant species.

Answer: We inserted the requested details (see lines 142 and 153-155 of the new version of the manuscript).

- Line 156: There is a typo, please replace "stinging nettle vs chaste tree" with "stinging nettle vs grapevine".

Answer: Done (see lines 170 of the new version of the manuscript).

- Lines 163-165: Was 20 H. obsoletus adults used per each cage? Please explain.
- Line 166: Again typo, please replace "or nettle and chaste tree" with "or nettle and grapevine"
- Line 167: Please give details on the size of the plants and source of the plants (from seeds, from nature...)

Answer: We are grateful to the reviewer for these comments allowing the improvement of the manuscript. According to the questions from line 163-165, 166 and 167, we changed the manuscript (see lines 146-151, 172 and 185-189 of the new version of the manuscript).

- Lines: 179-181: Please give more details on this experiment. If I understood correctly, the nettle was removed from the ditch at the time of the adult flight period; this needs to be better explained. In addition please give details on the size of the plants, distance between the plants within each group and size of the sticky traps used for the monitoring of the H. obsoletus adults. It seems that plants must have been fully grown to have enough canopies for sticky traps to be placed within. Please explain the source of plants and condition under which they were grown.

Answer: We inserted the details requested by the reviewer (see lines 194-197 and 201-205 of the new version of the manuscript).

- Lines 208-209: Please give details on the source of chest tree plants. Were they grown from seeds, or taken from nature and then potted. What was the size of the plants?

Answer: We inserted the details requested by the reviewer (see lines 214-215 and 218-219 of the new version of the manuscript).

- Lines 216-217: Please explain why limited number of insects was collected from experimental plants.

Answer: In the case of insects collected from the plant TBS5 we made a typing errors: collected insects were 10 and not 1. The limited number of insects collected after the transmission period was due to the fact that the body of some insects was strongly deteriorated and not suitable for the following molecular analysis (see lines 240 and Table 3 of the new version of the manuscript).

Discussion:

- Line 326: Typo, please replace "Serbia" with "Montenegro".

Answer: Done (see lines 350 of the new version of the manuscript).

- Please give and discuss precise context of the finding that St5 genotype is the one successfully transmitted to Vitex. St1, St2 and St30 (previously found associated with Vitex or transmitted by Vitex associated H. obsoletus; Kosovac et al., 2016) and St5 are of the same stamp Cluster b-II, with St5 differing only in 4nt from St2 which is transmitted to grapevine by naturally infected H. obsoletus originating from Vitex agnus-castus from Montenegro. This is especially indicative because St2 is possible natural genotype associated with Vitex, because Ho used in this transmission were collected on Vitex from natural habitat, hence not associated with agroecosystem.
- There is an additional epidemiological importance of the St5 genotype which you transmitted with Ho originating from Urtica to Vitex. It is relevant that this genotype is so far known to be associated only with Convolvulus as source plant, Hyalesthes obsoletus from Convolvulus as vectors, and from grapevine, in wide geographic area from Germany, Italy, Austria, Slovenia, to Macedonia (Pierro et al., 2018, Phytopathol). Also, it is one of the dominant stamp genotypes of the recent epidemics in Tuscany (Pierro et al., 2018, Ann App Biol). Hence, this strain was never associated with Urtica as host plant.

Please, give a short discussion on this finding and on the possibility that all other genotypes which are not transmitted to Vitex in your study could be (as expected) those that are strictly associated with Urtica (stamp a1 and a2 clusters) and that it is probably why they couldn't be transmitted. Of course, this all requires further (future) investigations, but it would be very informative and lucrative to discuss this situation in context of present findings.

Answer: We are grateful to the reviewer for these comments allowing the improvement of the discussion of the obtained results. We modified the discussion inserting new sentences focused on the points raised by the reviewer (see lines 350-357 and 369-371 of the new version of the manuscript).

Comments of Reviewers:
Reviewer 2:
The aim of this works was to verify if chaste tree is attractive for Hyalesthes obsoletus, the vector of Candidatus phytoplasma solani. In this work was show that the chaste tree plant can be host to both the insect vector H. obsoletus and the Ca. phytoplasma solani. However previous works, as indicated by the authors, have shown this although in separate papers. About the experiments to verify the attractiveness of chaste tree and grapevine for H. obsoletus from stinging nettle among the experiments conducted, some procedural gaps must be filled. In particular in the laboratory and semi-field experiments no phytosanitary status of the plants was indicated. It is not clear whether these plants have been tested for the presence of pathogens and / or phytoplasmas. This information is important because could be affect the volatiles elements from the plants. While in the transmission test was indicated that the plants, was PCR-negative to 'Ca. P. solani'. Furthermore, regarding to the test with the olfactometer some information about the instrument must be added. About the semi-field experiment, the study seem based on a low number of insects. Also, in the field experiments it is not indicated how many insects were analyzed in this experiment. However, the news of this work is that H. obsoletus was able to transmit from nettle to chaste tree.

See below for detail:

Introduction
Line 86: Add that several other insects are referred as suspected vectors of Ca. solani phytoplasma
<u>Answer</u>: We are grateful to the reviewer for these comments allowing the improvement of the introduction. We insert new references (see lines 83-91 of the new version of the manuscript).

Lines 115-116: 'In both olfactometric and field studies chaste tree resulted more attractive than grapevine for H. obsoletus adults (Sharon et al., 2005; Zahavi et al., 2007).'
Other more recent tests show a significant attraction of male H. obsoletus to chaste tree, and of the females to nettle (Riolo et al., 2012).
<u>Answer</u>: We insert the suggested citation (see lines 123-124 of the new version of the manuscript).

Line 124: 'Considering such contradictory data'
These are not contradictory data, it is better to write that the epidemiological cycle involving both, the plant insect vector and the pathogen has not yet been shown
<u>Answer</u>: Due to the modifications inserted throughout the introduction, we re-phrased the sentence by deleting the words "Considering such contradictory data" (see lines 98-99 of the new version of the manuscript).

Materials and Methods
Line 137: 'Survival of Hyalesthes obsoletus from stinging nettle on chaste tree and grapevine.'
Indicate on how many insects were tested in the survival experiment form each plant species
<u>Answer</u>: See answer Reviewer 1 (see lines 153-155 of the new version of the manuscript).

Line 155: 'choice test using two-choice olfactometer between shoots of chaste tree vs grapevine'
Provide more information about the type of olfactometer and the method used. In this experiment we indicated the females and males, however no data related to these aspects was included in the results. Indicate if the phytosanitary status of the plants used in this experiment has been evaluated
<u>Answer</u>: We improve the olfactometer description and the phytosanitary status of the plants (see lines 169-171 of the new version of the manuscript). Regarding the females and males data , males and females were considered: "To establish the proportion of males and females that were attracted by one of the two plants was different …" (see lines 174-175) and the results of these comparisons

were reported at lines 278–281. As reported at lines 160-162 of the old manuscript version males and females were not analysed separately for plant species preference, due to the low number of individuals that chose the plant and at the absence of differences in the choosing between males and females. At this purpose we added a new sentence in M&M at lines 176-179 to explain because the data of the two sexes were pulled.

Line 163: ‘Semi-field conditions’
In this experiment, the authors analyzing a very low number of insects. In fact most of these do not choose any plant (See Fig 3). Do you have evaluated the plant’s phytosanitary status? Specify better
Answer: Concerning the low number of insects analyzed in attractiveness trials in semi-field conditions, it is truth that a low number of insects selected the plants but the results are the average of three replications, representing robust data. Moreover, we indicated the phytosanitary condition of the plants (see lines 182-188 of the new version of the manuscript).

Line 176: ‘For each distance, 6 groups of the 3 plants were considered, the distance between each..’
From which plants each group was composed? Specify better
Answer: We modified the manuscript as suggested (see lines 198-199 of the new version of the manuscript).

Line 206: ‘Species recognition was confirmed based on the taxonomic keys by Bertin et al. (2010).
Why do you say it only at this point? not needed before
Answer: The species identification was done collecting randomly specimens throughout all bioassays. We move the citation Bertin et al. (2010) from Transmission trials paragraph to Survival of Hyalesthes obsoletus one as requested (see lines 155-156 of the new version of the manuscript).

Results
Line 248: ‘Attractiveness of chaste tree and grapevine for Hyalesthes obsoletus from stinging nettle’
How many insects was analyzed in this experiments? How was the analysis done? On the total number of insects, or on males and females separately? Specify.
Answer: Forty individuals (20 females and 20 males) were tested for each comparison. We changed the Materials and Methods of the manuscript in order to make it clear (see lines 171 of the new version of the manuscript). A G-test of goodness of fit was used putting together males and females (see lines 176 of the new version of the manuscript).

Lines 260-270: ‘The interactions time × plant, plant ×distance and time × plant × distance were significant due to the fact that the captures were influenced by time and distance only for stinging nettle and chaste tree (Table 1).
From the ANOVA table, this is not clear. It would be appropriate to show the data from which the ANOVA table was obtained.
Answer: The interpretation of the significance of the interactions is based on the fact that (i) there are significant differences both between dates and between distances, and (ii) these must necessarily be due to the nettle and Vitex, being the captures on vines equal to zero in the three samplings (time) and to three distances. To make the interpretation even more explicit, the sentence has been extended (see lines 294-295 of the new version of the manuscript)

Discussion
Lines 307-311: ‘Considering the two latter, as no captures occurred in grapevine, chaste tree seemed to be preferred. The higher attractiveness of chaste tree compared to grapevine was showed by semi-field experiments in which even chaste tree was significantly less attractive than stinging

nettle in only one of the two years. With reference to the two true host plants, i.e. stinging nettle and chaste tree, preference for the former may still be associated with the origin of adults used for the experiments. '

This paragraph is very confusing. What you want to explain that the most attractive species is the nettle followed by chaste tree while the less attractive was the grapevine species. This, results could be influenced from the origin of adults used for the experiments all collected from the nettle plants. Simplify.

Answer: We are grateful to the reviewer for these comments allowing the improvement of the manuscript, we rewrited the manuscript (see lines 331-336 of the new version of the manuscript).

Vitex agnus-castus cannot be used as trap plant for the vector *Hyalesthes obsoletus* to prevent infections by *'Candidatus* Phytoplasma solani' in northern Italian vineyards: experimental evidence

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Running title: Possible role of chaste tree in bois noir epidemiology

Abstract

Bois noir (BN), the ~~more widespread~~ disease of the grapevine yellows complex, ~~is causing~~ ~~a~~ considerable yield loss in vineyards. BN is associated with phytoplasma strains of the species ‘*Candidatus* Phytoplasma solani' (taxonomic subgroup 16SrXII-A). In Europe, BN phytoplasma is transmitted to grapevine mainly by *Hyalesthes obsoletus*, a polyphagous cixiid completing its life cycle on stinging nettle and field bindweed. Due to the complexity of BN epidemiology, no effective control strategies have been developed. In ~~east~~ Mediterranean coast of Israel, chaste tree (*Vitex agnus-castus*), even if found to be the preferred host plant of *H. obsoletus*, did not harbor BN phytoplasma. Thus, a "push and pull" strategy was suggested based on the fact that chaste tree plants located at vineyard borders was an effective trap plant for *H. obsoletus* adults. However, in east Adriatic coast of Montenegro, chaste tree was found to be a key source plant for BN phytoplasma transmission to grapevine. ~~Considering such contradictory data, this~~ study aimed to investigate (i) the interaction between chaste tree and *H. obsoletus* through survival, attractiveness and oviposition experiments conducted comparing the behavior of *H. obsoletus* in chaste tree versus stinging nettle and grapevine, and (ii) the capability of chaste tree to harbor ‘*Ca*. P. solani’ in northern Italy through transmission trials. Obtained data showed that (i) *H. obsoletus* adults can survive on chaste tree and grapevine even over a week; (ii) *H. obsoletus* adults prefer chaste tree to grapevine; (iii) *H. obsoletus* can produce eggs and overwinter as nymphs on chaste tree, even if at a lesser extent than on stinging nettle; (iv) *H. obsoletus* originating from nettle is able to transmit '*Ca*. P. solani' to chaste tree (two plants out of 16 were found infected by the BN phytoplasma strain St5 identified in *H. obsoletus* specimens). These results increased knowledge about the role of *Vitex agnus-castus* as host plant of *H. obsoletus* and BN phytoplasma in northern Italy and do not allow considering chaste tree as trap plant at vineyard borders.

Key words: insect vectors, Bois noir, trap plant, transmission trials, *stamp gene*

1 INTRODUCTION

Europe is the world leader in grape production with almost half of the global vi[illegible]rowing. Italy is the second top producer of grapes after China with about 8.2 million tons (FAO, 2016). Quality and quantity of ~~viticulture~~ production are damaged by a ~~wide~~ range of pathogens associated with diseases affecting the main cultivated grapevine varieties (Bellée et al., 2018). Among these diseases, the grapevine yellows (GY) complex is one of the most important threats to viticulture in many countries (Magarey, 2017). The GY causal agents are phytoplasmas ('*Candidatus* Phytoplasma'), ~~cell-wall less~~ obligate parasitic bacteria transmitted by insect vectors to plants, ~~in which they reside in phloem tissues~~ (Angelini et al., 2018). Interestingly, even if undistinguishable based on symptoms, the main diseases within the GY complex are associated with genetically distinct phytoplasmas, belonging to at least six '*Ca*. Phytoplasma' species, characterized by different biological features that reflect on disease epidemiological patterns (Belli et al., 2010; Angelini et al., 2018).

Bois noir (BN) is the most widespread disease of the GY complex in the Euro-Mediterranean area, where it may lead to a total yield loss and even grapevine death (Belli et al., 2010; Pavan et al., 2012). BN is associated with grapevine infection by phytoplasma strains (Bois noir phytoplasma strains, BNp) of the species '*Candidatus* Phytoplasma (*Ca*. P.) solani' (subgroup 16SrXII-A) (Quaglino et al., 2013). In the Euro-Mediterranean regions the main '*Ca*. P. solani' insect vector is *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) (Maixner, 1994; Sforza et al., 1998; Bressan et al., 2007), a polyphagous planthopper living preferentially on stinging nettle (*Urtica dioica* L.), field bindweed (*Convolvulus arvensis* L.), stinking hawk's-beard (*Crepis foetida* L.), and *Artemisia* spp. in and/or around vineyards (Alma et al., 1988; Sforza et al., 1998; Weber & Mainer, 1988; Langer & Maixner, 2004; Mori et al., 2008b, 2013; Cargnus et al., 2012; Kosovac et al., 2013). Recently, *Reptalus panzeri* Low (Homoptera: Cixiidae) has been reported as vector of '*Ca*. P. solani' (CaPsol) in Serbian vineyards (Cvrković et al., 2014) while *Macrosteles quadripunctulatus* (Kirschbaum) (Homoptera: Cicadellidae) was found able to transmit CaPsol to potted grapevine plants (Batlle et al., 2008). In addition *Anaceratagallia ribauti* (Ossiannilsson) (Homoptera: Cicadellidae) and *Reptalus quinquecostatus* (Dufour) (Homoptera: Cixiidae) were reported as vectors ~~even if not~~ to grapevine (Riedle-Bauer et al., 2008; Chuche et al., 2016). Other studies reported that different Cixiidae and Cicadellidae species have been captured within or near BN-diseased vineyards and found to contain CaPsol (Oliveri et al., 2015; Šafářová et al., 2018) but ~~such insects~~ are not currently considered ~~to be~~ involved in CaPsol transmission to grapevine.

~~The sequence~~ analysis of *tufB* gene revealed that two main '*Ca*. P. solani' *tuf*-types are present on grapevines and alternative plant hosts, according to diverse ecological pathosystems: (i) field bindweed - *H. obsoletus* - grapevine *tuf*-type b, (ii) stinging nettle - *H. obsoletus* - grapevine *tuf*-type a (Langer & Maixner, 2004). Recently, in Austria, Aryan et al. (2014) detected a large presence of a *tuf*-type b with a distinguished *Hpa*II-restriction profile designed as *tuf*-type b2 that appears to have different ecological features. Interestingly, ~~most~~ recent evidence highlighted the existence of a new BN epidemiological cycles of *tuf*-type b '*Ca*. P. solani' strain in the Balkan region and in east Adriatic coast of Montenegro, sourced respectively by *C. foetida* and *Vitex agnus-castus* L. transmitted by their associated *H. obsoletus* population (Kosovac et al., 2016, 2019). Moreover, several weeds, such as *Chenopodium album* L. and *Malva sylvestris* L., host the '*Ca*. P. solani' in or around infected vineyards and can therefore play a role in BN ~~spreading~~ (Marchi et al., 2015; Mori et al., 2015; Oliveri et al., 2015). Molecular epidemiology approaches, using *vmp1*- and *stamp*-based markers allowed knowledge ~~to be increased~~ of the populations of BN throughout vineyards and their surroundings in the Mediterranean area (Fialová et al., 2009; Fabre et al., 2011; Foissac et al., 2013; Murolo et al., 2014; Landi et al., 2015; Murolo & Romanazzi, 2015; Pierro et al., 2018a, 2018b).

The complexity of BN disease epidemiology renders it difficult to design efficient control strategies. Insecticides applied to the grapevine canopy influence neither the disease nor the presence of *H. obsoletus* (Maixner, 2007; Mori et al., 2008b). The management of *H. obsoletus* host plants in the vineyards and surrounding areas is therefore considered crucial for BN control (Maixner, 2010; Mori et al., 2012; Panassiti et al., 2017). Thus, preventive measures, such as checking the health status of propagation materials (i.e., mother plants and grafted cuttings) and treating of cuttings through thermotherapy, are applied to limit long distance dissemination and in-field spread of the disease (Mannini et al., 2007). Other strategies for reducing BN spread or incidence are based on (i) preventive removal of the grape suckers on which *H. obsoletus* could feed ~~after grass mowing~~ (Picciau et al., 2010); (ii) trunk cutting above the engagement point on symptomatic grapevines (Kast et al., 2008; Riedle-Bauer et al., 2010); (iii) treatments by resistance inducers (Romanazzi et al., 2009, 2013). ~~In prospective, also plan~~t volatiles from host plants can be used for reducing vineyard colonization by *H. obsoletus* (Riolo *et al*., 2017).

In Israel, chaste tree (*Vitex agnus-castus* L.) is a plant where *H. obsoletus* can complete its life cycle (Sharon et al., 2005). In both olfactometric and field studies chaste tree ~~resulted~~ more attractive than grapevine for *H. obsoletus* adults (Sharon et al., 2005; Zahavi et al., 2007; Riolo et al., 2012). Therefore, a "push and pull" strategy based on the use of chaste tree as trap plant at vineyard borders to reduce the vector population ~~living~~ inside ~~the vineyards~~ was suggested (Zahavi et al., 2007). The ~~validity of this~~ strategy ~~is~~ reinforced by the fact that in Israel chaste tree was never found infected by '*Ca*. P. solani' and thus cannot serve as an inoculation source for grapevine (Sharon et al., 2015).

This study aimed to investigate the ~~possible~~ role of *V. agnus-castus* as host plant of *H. obsoletus* and CaPsol in northern Italy. ~~In detail, the~~ interaction between chaste tree and *H. obsoletus* was examined through survival, attractiveness and oviposition trials, while the capability of chaste tree to harbor CaPsol in northern Italy was studied through transmission trials in controlled conditions. In these studies, *H. obsoletus* adults collected on stinging nettle were used because this plant is the most important external source of infected vectors for Northern Italian vineyards (Mori et al., 2008b, 2015) and therefore the possibility of using chaste tree as trap plant at vineyard borders must be evaluated on this population.

2 MATERIAL AND METHODS

2.1 Survival of *Hyalesthes obsoletus* from stinging nettle on chaste tree and grapevine

Hyalesthes obsoletus adults were collected by using a sweep net and pooter in Veneto region on 4th July 2016 and 27th June 2017 from stinging nettle plants, growing along a ditch bordering a BN infected vineyard (45°23’32.42’’N; 11°09’45.62’’E), and were maintained for ten days under controlled conditions [25±3 °C, 70±5 RH, 16:8 (L:D) daily light cycle] in ~~insects proof~~ cages on potted plants of chaste tree, stinging nettle and grapevine. The chaste tree plants were generated by tissue culture in Guagno nursery (Padova, Italy), stinging nettle plants were taken from field, and grapevine plants were one-year Chardonnay grafted on SO4 rootstock in Vivai Cooperativi Rauscedo (Pordenone, Italy). The plants, grown in 5 L pots, were in good vegetative condition and did not show ~~any~~ symptoms ~~related to~~ biotic and abiotic stresses. The three plant species had similar volume and leaf density (diameter about 0.3 m and ~~high about~~ 0.8 m).
Both years, the *H. obsoletus* individuals, collected from stinging nettle, were randomly confined on 8 singularly caged potted plants ~~per~~ each of the three host species. On average, in 2016 were used 28.5, 14.1, and 25.1 adults on chaste tree, stinging nettle and grapevine, respectively; in 2017, were used 14.6, 15.3, and 17.6 adults on chaste tree, stinging nettle and grapevine, respectively. Species ~~recognition~~ was confirmed based on the taxonomic keys by Bertin et al. (2010).

During the 10-day confinement, the number of dead individuals was counted daily. On the last sampling day the number of alive individuals was also counted, to ~~know~~ the total number in each cage. Kaplan-Meier analysis was used to estimate the survival curve on the three plants and the comparison between two survival curves was made by the log-rank test.

2.2 Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle

The attractiveness of chaste tree for *H. obsoletus* collected on stinging nettle was evaluated under laboratory, semi-field and field conditions.

~~(i) Laboratory conditions: the~~ experiment was conducted in 2017 using *H. obsoletus* adults captured on stinging nettle (see survival trials §). Before their use in the experiment the adults were left on Petri dishes with water for 12 hours. The planthoppers then underwent a choice test using a custom made two-choice olfactometer [following Dicke et al. (1988)] between shoots of chaste tree vs grapevine (cv Chardonnay), chaste tree vs stinging nettle, stinging nettle vs grapevine. The shoots were in good ~~vegetative~~ condition and did not show any symptoms ~~related to~~ biotic and abiotic stresses. Forty individuals (20 females and 20 males) were tested for each comparison. If 10 minutes after positioning the insect was still at the start on the olfactometer, the test was considered as "No choice". Data analysis was performed on the individuals that chose one of the two plants under comparison. To establish if the proportion of males and females that were attracted by one of the two plants was different, a Fisher's exact test was used. To ~~know~~ if one plant was preferred by adults more than the other in comparison, a G-test of goodness of fit was used. Since the percentages of males and females who have chosen one of the two plants under comparison are always differed for no more than 7%, this last analysis was conducted pooling ~~together~~ the adults of the two sexes.

~~(ii) Semi-field conditions: in~~ 2016 and 2017, 9 cages (0.5 m × 0.5 m × 1.0 m) containing potted plants of two species, namely chaste tree and grapevine (n. 3 cages) or chaste tree and nettle (n. 3 cages) or nettle and grapevine (n. 3) were prepared. The origin and the vegetative status of the plants were the same of those used in the survival experiment (see 2.1 §). The plants of the two species under comparison inside each cage were pruned to similar volume and leaf density (diameter of about 0.3 m and high of about 0.5 m). In each cage 20 *H. obsoletus* adults (10 females and 10 males) (captured on stinging ~~nettle, see survival trials §~~) were confined. Cages were maintained under controlled conditions [25±3 °C, 70±5 RH, 16:8 (L:D) daily light cycle]. Observation of adult insect's position was done 1, 4 and 8 hours after caging. If the insect was on the net or on the bottom of the cage, the position was considered ~~as~~ "No choice". Data analysis was performed on the individuals that chose one of the two plants under comparison using a paired-sample *t* tes

~~(iii) Field conditions: the trial was~~ conducted in 2017 in a ploughed field (~~3.7 ha surface,~~ (45°23'34.92''N; 11°09'39.10''E) with one side (103 m long) bordered by a ditch covered with stinging nettle harbouring large *H. obsoletus* populations. At the time of the adults' flight period the stinging nettle along the ditch was mowed. Potted chaste tree, grapevine and nettle plants (see surviv rials §) were placed in the field at 5, 10 and 20 m from the border ~~in the same day of the stinging nettle mowing~~. For each distance, 6 groups of the 3 plants were considered, one for each of the three species. The distance between each plant group was 15 m and 1.0 m between each plant within the group. All potted plants of the three species under comparison were pruned to similar volume and leaf density (diameter of about 0.4 m and high of about 0.9 m) and irrigated twice a week. The plants were in good vegetative condition and did not show any symptoms ~~related to~~ biotic and abiotic ~~stresses~~. The presence of *H. obsoletus* adults on the three potted-plant species was monitored after nettle mowing by transparent ky traps (~~A5 paper size~~ 148 × 210 mm) positioned within their canopy. The number of individuals captured during the first and second week was counted. To compare field-trial data (number of *H. obsoletus* adults captured), a three-way ANOVA was used, considering ~~as source of variation~~ sampling time (first and second week from stinging nettle mowing), host plant (stinging nettle, grapevine and chaste tree) and distance from *H. obsoletus* source (5 m, 10 m and 20 m). Prior to analysis data normality was tested with the Shapiro–Wilk test, homogeneity was tested with Levene's variance test, the presence of outliers was assessed, and the data were log(x+1) transformed. For *post hoc* comp ons of means, LSD5% (least significant difference between two means at the 5% level) was used.

2.3 Egg laying of *Hyalesthes obsoletus* from stinging nettle on chaste tree

Insects proof cages (0.5 m × 0.5 m × 1.0 m) were arranged on potted plants of chaste tree (grown from tissue culture Guagno nurseries – Padova) and ~~stinging nettle (taken from nature)~~. Four and eight potted plants for each species were considered in 2016 and 2017 respectively. The pots had 50 L of capacity and the holes at the bottom were closed with insect-proof net to allow water flow but prevent the hatched-nymphs escaping, ~~The plants size was about~~ 0.4 m in diameter and ~~about~~ 0.9 m in height.

In each cage 100 *H. obsoletus* adults (50 females and 50 males), collected on stinging nettle (see su al trials §) on 21st July 2016 and 14th July 2017, were confined with the plants. The cages were maintained in an open field during winter. In February 2017 and 2018, *H. obsoletus* nymphs were extracted from the soil by Berlese funnel and analysed under stereomicroscope. Nymphs were identified using the dichotomous keys of Cargnus et al. (2012). Data collected in the two years were analysed together using a paired-sample *t*-test.

2.4. Transmission trials of BN phytoplasmas to chaste tree

In 2017 adults of *H. obsoletus* were collected on stinging nettle in a ditch bordering two BN-affected vineyards in Lombardy (Brescia province: 45°35'37.72''N; 10°09'33.36''E) and Veneto (Verona province: 45°23'32.42''N; 11°09'45.62''E) regions. Capturing of adults was done by using a sweep net and pooter. The captured insects were kept in jars for transport to the laboratory. The transmission trials were conducted with ~~twenty four~~ chaste tree plants, tested PCR-negative for '*Ca.* P. solani' in a greenhouse under controlled conditions (25±3 °C, 70±5 RH) located in Verona province (45°20'13.72''N; 11°13'03.28''E). The plants were singularly caged and divided into three groups: (i) plants TBS1-TBS8, with confined *H. obsoletus* individuals collected in Brescia (30 adults per plant), (ii) plants TVR1-TVR8, with confined *H. obsoletus* individuals collected in Verona (30 adults per plant), and (iii) plants T1-T8, without insects (control plants). Transmission trials were ~~left till the~~ end of adult survival. After this period, the plants were kept in an insect-free greenhouse.

Dead insects (136 in plants TBS1-TBS8; 146 in plants TVR1-TVR8), collected from the end of June till mid-July 2017, were stored in absolute ethanol at 4 °C. '*Ca.* P. solani' was ~~detected~~ by nested PCR-based amplification of *stamp* gene (Fabre et al., 2011) using ~~as templates~~ the total nucleic acids extracted from both the individual insect specimens (Marzachì et al., 1998) and the leaves of chaste tree plants (Angelini et al., 2001) collected in October 2017 and 2018. The plants were kept in an insect-free greenhouse for the ~~whole~~ transmission period. ~~Amplification~~ products were analyzed by electrophoreses in 1% agarose gel stained with Midori green under a UV transilluminator.

PCR products (StampF1/StampR1), amplified from insect adults and chaste tree samples, were sequenced in both strands (Sanger method, 5X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pair in the software BioEdit, version 7.2.6 (Hall, 1999). Obtained *stamp* gene nucleotide sequences were aligned using the ClustalW Multiple Alignment program in the software BioEdit and analysed by Sequence Identity Matrix to estimate ~~their~~ genetic diversity. *Stamp* sequence variants, identified in the study, were aligned and compared with representative sequences of previously defined sequence variants (Pierro et al., 2018a, 2018b); a nucleotide sequence identity of 100% was necessary for the attribution to such sequence variants.

3 RESULTS

3.1 Survival of *Hyalesthes obsoletus* from stinging nettle on chaste tree and grapevine

~~The data gathered in the two years showed that adults~~ of *H. obsoletus* collected from stinging nettle can survive on chaste tree and grapevine for some days, but the survival curves were significantly worse than those on stinging nettle (2016: grapevine vs stinging nettle, $X^2 = 251.4$, $p > 0.0001$; chaste tree vs stinging nettle, $X^2 = 104.6$, $p < 0.0001$; 2017: grapevine vs stinging nettle, $X^2 = 151.2$, $p > 0.0001$; chaste tree vs stinging nettle, $X^2 = 66.6$, $p < 0.0001$) (Figure 1). Survival on chaste tree and grapevine fell below 50% after three days in 2016 and after 5 days in 2017. In 2016 the survival curve on chaste tree was significantly better than on grapevine ($X^2 = 4.87$, $p = 0.016$), but in 2017 ~~this difference was not confirmed~~ ($X^2 = 1.47$, $p = 0.16$).

3.2 Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle

In the laboratory experiment with two-choice olfactometer, the proportion of males and females that chose one of the two plants under comparison with “no-choice” individuals was not significantly different ($p = 0.10$ for grapevine vs chaste tree, $p = 1$ for chaste tree vs stinging nettle, $p = 1$ for grapevine vs stinging nettle, Fisher’s Exact Test). *Hyalesthes obsoletus* adults did not show any significant preference for grapevine vs chaste tree ($G = 0.081$, $p = 0.78$), chaste tree vs stinging nettle ($G = 0.081$, $p = 0.78$) or grapevine vs stinging nettle ($G = 2.19$, $p = 0.14$) (Figure 2).

In the semi-field experiment, there were significant differences in the choice of plant species by *H. obsoletus* adults collected on stinging nettle (Figure 3). In particular, chaste tree was significantly preferred to grapevine in both 2016 ($t = 2.80$, d.f. = 8, $p = 0.02$) and 2017 ($t = 2.80$, d.f. = 8, $p = 0.02$); stinging nettle was significantly preferred to grapevine in ~~both~~ 2016 ($t = 3.39$, d.f. = 8, $p = 0.0095$) and 2017 ($t = 5.58$, d.f. = 8, $p = 0.0005$); stinging nettle was significantly preferred to chaste tree in 2017 ($t = 2.44$, df = 8, $p = 0.04$), but not in 2016 ($t = 1.42$, d.f. = 8, $p = 0.19$).

In the open field, captures of *H. obsoletus* from stinging nettle plants along a ditch were significantly influenced by time (i.e., days from nettle mowing), plants and distance from *H. obsoletus* adults’ source (Table 1). In particular, captures were higher the second than the first week from nettle mowing. On stinging nettle the captures were significantly higher than on the other two plants (Table 2). Although no ~~individual was~~ captured on grapevine, the differences with respect to chaste tree were not statistically significant ~~based on LSD5%~~ (Table 2). The captures decreased with ~~the increase of~~ distance from the ditch, i.e. from the source of *H. obsoletus* adults, and were significantly higher at 5 m than both 10 m and 20 m (Table 2). The interactions time × plant, plant × distance and time × plant × distance were significant ~~due to the fact that the~~ captures were influenced by time and distance only for stinging nettle and chaste tree, because on grapevines the captures were always zero (Table 1).

3.3 Egg laying of *Hyalesthes obsoletus* from stinging nettle on chaste tree

Based on the nymphs observed in February of the next year, *H. obsoletus* females laid eggs on potted plants in 10 out of 12 cages. Nymphs were recorded on the roots of both stinging nettle and chaste tree, showing indirectly that females had laid eggs on both plants, but a significantly higher number was observed on the former (t = 3.36, d.f. = 9; p = 0.009) (Figure 4).

3.4 Transmission trials

The PCR analyses for amplification of the *stamp* gene, performed on the total nucleic acids extracted from the chaste tree plants used in the transmission trials, showed the presence of '*Ca*. P. solani' in two plants (TBS6 and TBS7) ~~out~~ of 16 (12.5%). No amplification was observed in the other 14 chaste tree plants, on which insects were maintained, and on the eight control plants (without insects) (Table 3). The molecular analyses performed on the insect individuals collected from plants TBS6 and TBS7 revealed that five individuals out of 16 (31.25%) and six out of 18 (33.33%), respectively, were ~~found to be~~ infected by '*Ca*. P. solani'. *H. obsoletus* adults, collected from the 14 chaste tree plants negative to phytoplasma presence, were found to be infected at a percentage varying from 0 to 50% (Table 3). Nucleotide sequence analyses of the *stamp* gene showed that chaste tree plants and insect individuals feeding on them harboured the same '*Ca*. P. solani' strain, characterized by the *stamp* gene sequence variant St5.

The PCR analyses performed on chaste tree leaves collected in October 2018 (one year after the transmission trials), showed ~~that~~ all 24 chaste tree plants, including TBS6 and TBS7 (positive in 2017), were ~~negative to phytoplasma presence~~ (Table 3).

DISCUSSION

Survival of *H. obsoletus* adults from stinging nettle was better on the plants on which the nymphs developed (i.e. stinging nettle), than on ~~the other plants (i.e.~~ grapevine and chaste tree~~)~~. This occurrence was previously observed for *H. obsoletus* from stinging nettle or bindweed that had better survival on the origin plant than on the other (Mori et al., 2008; Kessler et al., 2011; Maixner et al., 2014). Survival on chaste tree was significantly better than on grapevine in one of the two study years. However, the differences were not so high as could be expected from the fact that chaste tree, unlike grapevine, is a true host of the planthopper (Sharon et al., 2015). Our study also indirectly confirmed that *H. obsoletus* can complete its life cycle on chaste tree because nymphs were observed in February on the roots of potted chaste tree plants on which planthopper adults had been caged and ~~been~~ able to lay eggs in the previous summer.

In the field, *H. obsoletus* adults from stinging nettle were more attracted by stinging nettle than chaste tree and ~~even~~ not captured on grapevine. Semi-field experiments confirmed both the scarce attractiveness of grapevine and the preference for stinging nettle than chaste tree. With reference to the two true host plants, namely stinging nettle and chaste tree, preference for the former may be associated with the origin of adults used for the experiments, all collected from stinging nettle plants. Based on this result, even the higher attractiveness of chaste tree in comparison with other plants observed in the olfactometer studies by Sharon et al. (2005) may have been influenced by the fact that most of the adults had been collected on chaste tree. The fact that chaste tree resulted significantly more attractive than grapevine would suggest its use as trap plant at vineyard borders. However, since the infected *H. obsoletus* adults that colonize vineyards in northern Italy move mostly from stinging nettle and for this planthopper population the nettle was more attractive than chaste tree, the use of healthy potted plants of stinging nettle as trap plants would be preferable. Our two-choice olfactometric studies showed no significant preference by *H. obsoletus* for either of the two plants, even if fewer adults were observed on grapevine than stinging nettle.

Results of the transmission trials conducted in the study ~~proved~~ that chaste tree can harbour '*Ca*. P. solani' and that infectious *H. obsoletus* adults from stinging nettle can ~~inoculate this phytoplasma in chaste tree~~. This evidence is in agreement with the results obtained by Kosovac et al. (2016), who demonstrated that ~~chaste tree naturally occurring~~ in vineyard agro-ecosystems in Montenegro ~~is~~ infected by '*Ca*. P. solani'. The '*Ca*. P. solani' strain St5, transmitted with *H. obsoletus* originating from stinging nettle to chaste tree in the present study, is ~~so far~~ known to be associated only with bindweed as source plant, *H. obsoletus* from bindweed as vector, and grapevine ~~in wide geographic European areas~~ (Pierro et al., 2018). Moreover, strain St5 groups within the bindweed-related *stamp* phylogenetic Cluster b-II along with strains St1, St2, and St30, previously ~~found~~ associated with chaste tree or transmitted to grapevine by chaste tree associated *H. obsoletus* (Kosovac et al., 2016). Thus, this is the first report of strain St5 transmitted to chaste tree by *H. obsoletus* from stinging nettle. As chaste tree constitutes an important reservoir for *H. obsoletus*-mediated transmission of BN phytoplasma to grapevine (Kosovac et al., 2016), our findings that chaste tree can host the '*Ca*. P. solani' strain St5, largely prevalent in the Franciacorta area, open a new intriguing scenario on its possible role in BN epidemiology in north Italy. ~~On the contrary, these~~ results are in disagreement with Sharon et al. (2005, 2015), who showed that, even if ~~it is~~ a preferred host plant of *H. obsoletus*, chaste tree did not harbour '*Ca.* P. solani'. Interestingly, ~~even if~~ '*Ca*. P. solani'-infected insect individuals were found on 15 ~~out~~ of 16 chaste tree plants used in transmission trials, *H. obsoletus* was only able to transmit the pathogen in two cases. This could be explained considering the short survival of insect adults on chaste tree; in fact, the insect populations decreased dramatically in 4 to 6 days after release. However, adults of *H. obsoletus* from stinging nettle survive on grapevine no better than on chaste tree and still are able to inoculate the BN phytoplasma. Moreover, the success of transmission trials can depend on the phytoplasma strain and ~~its~~ titer within the insect adults. For example, it is reasonable to hypothesize that '*Ca*. P. solani' strains not transmitted to chaste tree in the present study could be (as expected) those that are strictly associated with stinging nettle (*stamp* clusters a1 and a2). The fact that chaste tree plants, found positive for phytoplasma presence in October 2017, were phytoplasma-free in October 2018 can be explained by natural recovery from infection, as reported for a broad range of polyannual plants infected by phytoplasmas (Osler et al., 1993; Romanazzi et al., 2009), increased by abiotic stresses due to the overgrowth of chaste trees in pots under controlled conditions, which is not convenient in terms of spacing.

According to Sharon et al. (2005, 2015), showing that chaste tree is a preferred host plant of *H. obsoletus* and does not harbour '*Ca.* P. solani', in Israel a 'push & pull' strategy was suggested to reduce the population of *H. obsoletus* in a vineyard by using chaste tree as a trap plant (Zahavi et al., 2007). On the contrary, based on the findings of this and previous research work (Kosovac et al., 2016), it is doubtful that chaste tree can be used in the containment of the BN spread in Europe by using it as an attractant to *H. obsoletus* since it can also act as a reservoir of '*Ca*. P. solani'. However, volatiles from both chaste tree and stinging nettle could be used in the context of 'push & pull strategies' (Riolo et al., 2017).

In conclusion, ~~the results obtained increased the~~ knowledge about the role of *V. agnus-castus* as host plant of *H. obsoletus* and '*Candidatus* Phytoplasma solani' in north Italy. Further studies are needed to determine the actual role of chaste tree in ~~the~~ BN epidemiology.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interests.

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TABLE 1 Results of ANOVA on the captures of *H. obsoletus* recorded in the field on three potted plants (i.e., grapevine, chaste tree and stinging nettle) at two different times after stinging nettle mowing (1st and 2nd week) and at three different distances from the ditch source of the *H. obsoletus* adults (5 m, 10 m, 20 m).

Source of variation	**F**	**df**	**P**
Time	19.88	1, 90	< 0.0001
Plant	59.50	2, 90	< 0.0001
Distance	50.04	2, 90	< 0.0001
Time × plant	9.11	2, 90	< 0.0001
Time × distance	2.28	4, 90	0.10
Plant × distance	19.00	4, 90	< 0.0001
Time × plant × distance	3.55	4, 90	0.010

TABLE 2 erage capture recorded on the three plants and at the three different distances from the ditch where stinging nettle was mowed. SED, standard error of the differences between two means; LSD 5%, least significant difference between two means at P = 0.05; d.f., degrees of freedom associated with LSDs and SEDs.

Plant	**Mean**	**Mean [log (x+1)]**	**Distance**	**Mean**	**Mean [log (x+1)]**
Stinging nettle	5.72	(0.50)	5 m	5.64	(0.48)
Chaste tree	1.00	(0.13)	10 m	0.94	(0.12)
Grapevine	0.00	(0.00)	20 m	0.14	(0.03)
Stinging nettle vs chaste tree			5 m vs 10m		
SED		(0.15)			(0.15)
LSD 5%		(0.30)			(0.31)
d.f.		34			34
Chaste tree vs grapevine			10 m vs 20 m		
SED		(0.07)			(0.08)
LSD 5%		(0.15)			(0.16)
d.f.		34			34

TABLE 3 Results of transmission trials conducted using *H. obsoletus*, collected on sting nettle bordering BN-infected vineyards, and PCR-negative for '*Ca*. P. solani' chaste tree plants.

Origin	**Plants**			**Insects**			
	#	**BNp-infected (strain)**		**Number**			**BNp strain**
		Oct 17	**Oct 18**	**Released**	**Collected**	**BNp-infected**	
Verona	TVR1	-	-	30	27	10 (37%)	
	TVR2	-	-	30	13	3 (23%)	
	TVR3	-	-	30	21	10 (48%)	
	TVR4	-	-	30	20	4 (20%)	
	TVR5	-	-	30	15	4 (27%)	
	TVR6	-	-	30	19	3 (16%)	
	TVR7	-	-	30	16	4 (25%)	
	TVR8	-	-	30	15	1 (7%)	
Brescia	TBS1	-	-	30	17	4 (23%)	
	TBS2	-	-	30	18	3 (17%)	
	TBS3	-	-	30	17	8 (47%)	
	TBS4	-	-	30	20	2 (10%)	
	TBS5	-	-	30	10	0	
	TBS6	+ (St5)	-	30	16	5 (31%)	St5
	TBS7	+ (St5)	-	30	18	6 (33%)	St5
	TBS8	-	-	30	20	10 (50%)	
Control	T1	-	-				
	T2	-	-				
	T3	-	-				
	T4	-	-				
	T5	-	-				
	T6	-	-				
	T7	-	-				
	T8	-	-				

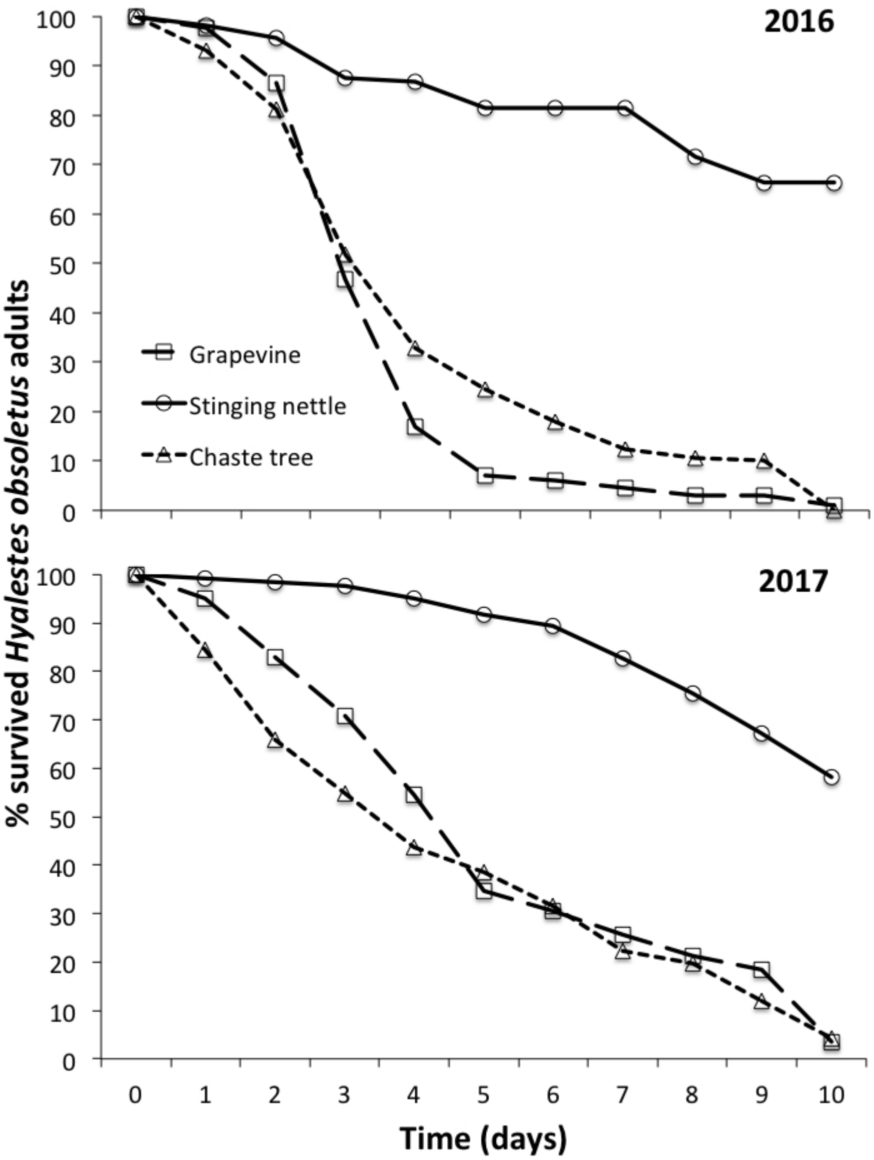

FIGURE 1 Survival of H. obsoletus from stinging nettle recorded in 2016 and 2017 on three different plant species.

254x338mm (72 x 72 DPI)

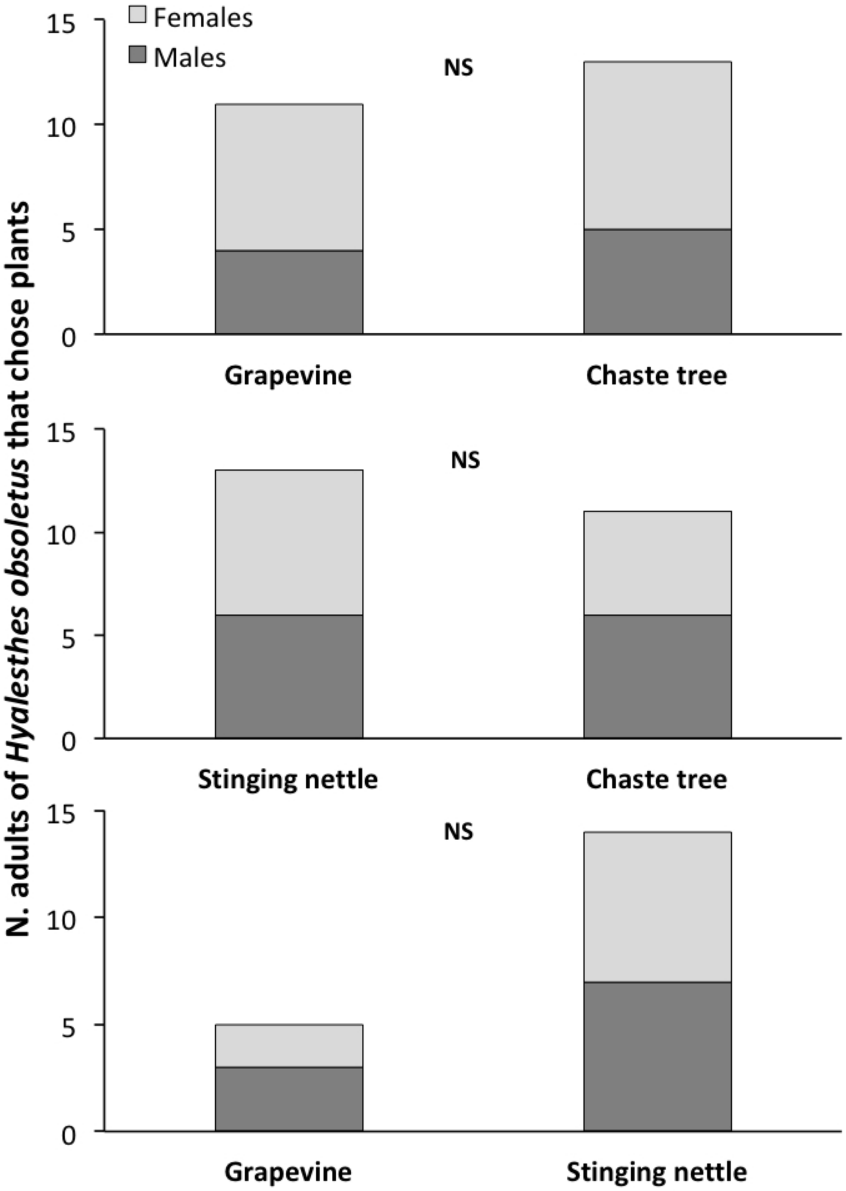

FIGURE 2 Number of H. obsoletus individuals (males and females) out of 40 that moved towards the two plants under comparison in two-choice olfactometer tests. NS indicates not significant differences (α = 0.05) with G-test of goodness of fit.

254x338mm (72 x 72 DPI)

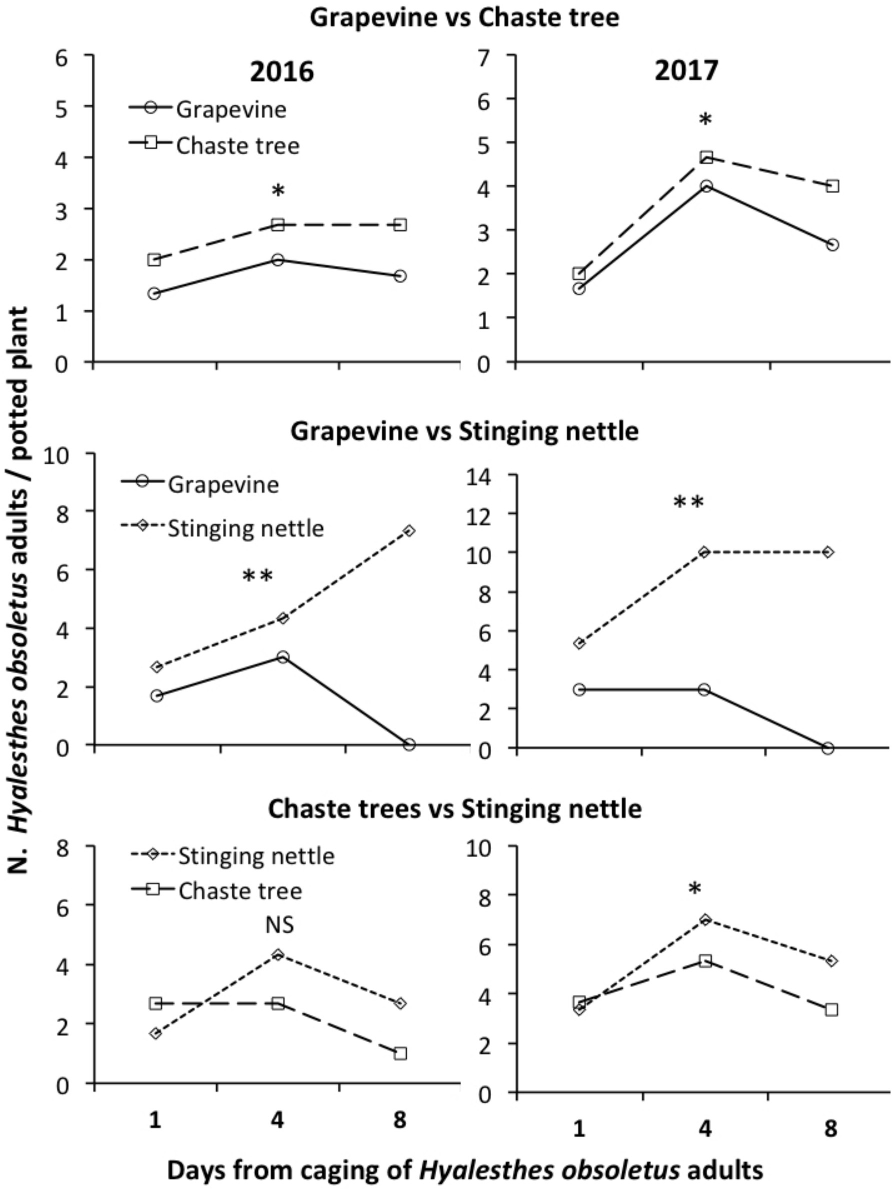

FIGURE 3 Number of H. obsoletus individuals out of 60 choosing the different plants in two-choice test with potted plants. NS, * and ** indicate, respectively, not significant and significant differences according to a paired-sample t test (α = 0.05 and 0.01).

254x338mm (72 x 72 DPI)

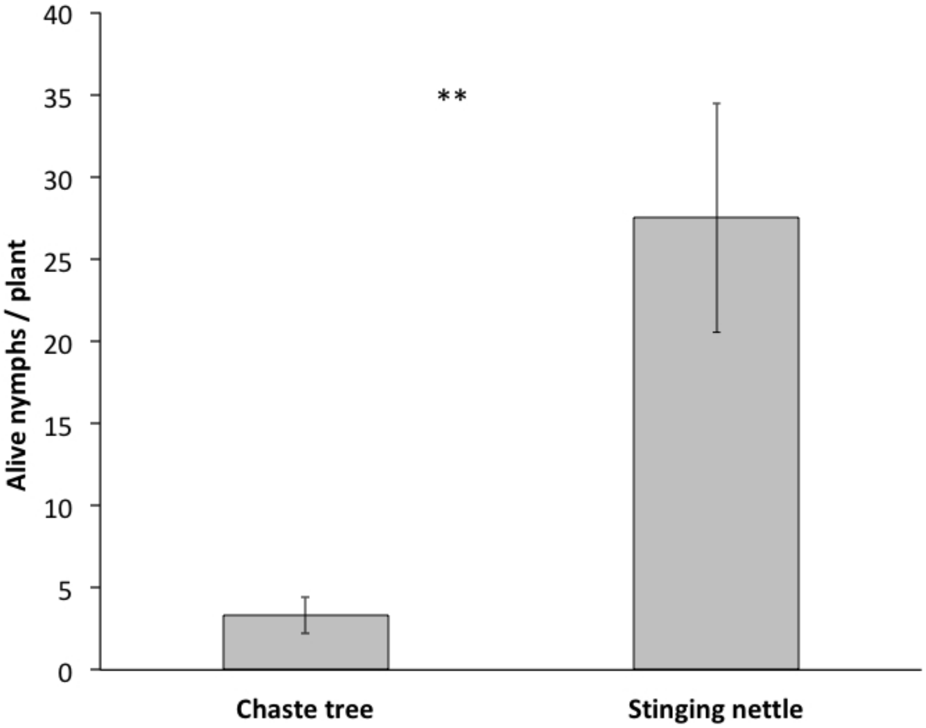

FIGURE 4 Alive nymphs of H. obsoletus observed on the roots of the two plant species in the February following the oviposition period in late summer of the previous year. ** = significant differences according to a paired-sample t test (α = 0.01).

254x190mm (72 x 72 DPI)

Vitex agnus-castus cannot be used as trap plant for the vector *Hyalesthes obsoletus* to prevent infections by '*Candidatus* Phytoplasma solani' in northern Italian vineyards: experimental evidence ~~Can *Vitex agnus-castus* to be used as trap plant for the vector *Hyalesthes obsoletus* to prevent infections by '*Candidatus* Phytoplasma solani' in Northern Italy vineyards?~~

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Running title: Possible role of chaste tree in bois noir epidemiology

Abstract

Bois noir (BN), the more widespread disease of the grapevine yellows complex, is causing a considerable yield loss in vineyards. BN is associated with phytoplasma strains of the species ‘*Candidatus* Phytoplasma solani' (taxonomic subgroup 16SrXII-A). In Europe, BN phytoplasma is transmitted to grapevine mainly by *Hyalesthes obsoletus*, a polyphagous cixiid completing its life cycle on stinging nettle and field bindweed. Due to the complexity of BN epidemiology, no effective control strategies have been developed. In east Mediterranean coast of Israel~~Israel~~, chaste tree (*Vitex agnus-castus*), even if found to be the preferred host plant of *H. obsoletus*, did not harbor BN phytoplasma. Thus, a "push and pull" strategy was suggested based on the fact that chaste tree plants located at vineyard borders was an effective trap plant for *H. obsoletus* adults. However, in east Adriatic coast of Montenegro~~Eastern Europe~~, chaste tree was found to be a key source plant for BN phytoplasma transmission to grapevine. Considering such contradictory data, this study aimed to investigate (i) the interaction between chaste tree and *H. obsoletus* through survival, attractiveness and oviposition experiments conducted comparing the behavior of *H. obsoletus* in chaste tree versus stinging nettle and grapevine, and (ii) the capability of chaste tree to harbor ‘*Ca*. P. solani’ in n~~N~~orthern Italy through transmission trials. Obtained data showed that (i) *H. obsoletus* adults can survive on chaste tree and grapevine even over a week; (ii) *H. obsoletus* adults prefer chaste tree to grapevine; (iii) *H. obsoletus* can produce eggs and overwinter as nymphs on chaste tree, even if at a lesser extent than on stinging nettle; (iv) *H. obsoletus* originating from nettle is able to transmit '*Ca*. P. solani' ~~from nettle~~ to chaste tree (two plants out of 16 were found infected by the BN phytoplasma strain St5 identified in *H. obsoletus* specimens). These results increased knowledge about the role of *Vitex agnus-castus* as host plant of *H. obsoletus* and BN phytoplasma in n~~N~~orthern Italy and do not allow considering chaste tree as trap plant at vineyard borders.

Key words: insect vectors, Bois noir, trap plant, transmission trials, *stamp gene*

1 INTRODUCTION

Europe is the world leader in grape production with almost half of the global vine-growing. Italy is the second top producer of grapes after China with about 8.2 million tons (FAO, 2016). Quality and quantity of viticulture production are damaged by a wide range of pathogens associated with diseases affecting the main cultivated grapevine varieties (Bellée et al., 2018). Among these diseases, the grapevine yellows (GY) complex is one of the most important threats to viticulture in many countries (Magarey, 2017). The GY causal agents are phytoplasmas ('*Candidatus* Phytoplasma'), cell-wall less obligate parasitic bacteria transmitted by insect vectors to plants, in which they reside in phloem tissues (Angelini et al., 2018). Interestingly, even if undistinguishable based on symptoms, the main diseases within the GY complex are associated with genetically distinct phytoplasmas, belonging to at least six '*Ca*. Phytoplasma' species, characterized by different biological features that reflect on disease epidemiological patterns (Belli et al., 2010; Angelini et al., 2018).

Bois noir (BN) is the most widespread disease of the GY complex in the Euro-Mediterranean area, where it may lead to a total yield loss and even grapevine death (Belli et al., 2010; Pavan et al., 2012). BN is associated with grapevine infection by phytoplasma strains (Bois noir phytoplasma strains, BNp) of the species '*Candidatus* Phytoplasma (*Ca*. P.) solani' (subgroup 16SrXII-A) (Quaglino et al., 2013). In the Euro-Mediterranean regions the main '*Ca*. P. solani' insect vector is *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) (Maixner, 1994; Sforza et al., 1998; Bressan et al., 2007), a polyphagous planthopper living preferentially on stinging nettle (*Urtica dioica* L.), field bindweed (*Convolvulus arvensis* L.), stinking hawk's-beard (*Crepis foetida* L.), and *Artemisia* spp. in and/or around vineyards (Alma et al., 1988; Sforza et al., 1998; Weber & Mainer, 1988; Langer & Maixner, 2004; Mori et al., 2008b, 2013; Cargnus et al., 2012; Kosovac et al., 2013). Recently, *Reptalus panzeri* ~~(~~Low~~)~~ (Homoptera: Cixiidae) has been reported as vector of '*Ca*. P. solani' (CaPsol) in Serbian vineyards (Cvrković et al., 2014) while ~~,~~ *Macrosteles quadripunctulatus* (Kirschbaum) (Homoptera: Cicadellidae) was found able to transmit CaPsol to potted grapevine plants (Batlle et al., 2008). In addition ~~while~~ *Anaceratagallia ribauti* (Ossiannilsson) (Homoptera: Cicadellidae) and *Reptalus quinquecostatus* (Dufour) (Homoptera: Cixiidae) were reported as vectors even if not to grapevine (Riedle-Bauer et al., 2008; Chuche et al., 2016). Other studies reported that different Cixiidae and Cicadellidae species have been captured within or near BN-diseased vineyards and found to contain CaPsol (Oliveri et al., 2015; Šafářová et al., 2018) but such insects are not currently considered to be involved in CaPsol transmission to grapevine.

The sequence analysis of *tufB* gene revealed that two main '*Ca*. P. solani' *tuf*-types are present on grapevines and alternative plant hosts, according to diverse ecological pathosystems: (i) field bindweed - *H. obsoletus* - grapevine *tuf*-type b, (ii) stinging nettle - *H. obsoletus* - grapevine *tuf*-type a (Langer & Maixner, 2004). Recently, in Austria, Aryan et al. (2014) detected a large presence of a *tuf*-type b with a distinguished *Hpa*II-restriction profile designed as *tuf*-type b2 that appears to have different ecological features. Interestingly, most recent evidence highlighted the existence of a new BN epidemiological cycles of *tuf*-type b '*Ca*. P. solani' strain in the Balkan region and in east Adriatic coast of Montenegro, sourced respectively by *C. foetida* and *Vitex agnus-castus* L. transmitted by ~~its~~ their associated *H. obsoletus* population (Kosovac et al., 2016, 2019). Moreover, several weeds, such as *Chenopodium album* L. and *Malva sylvestris* L., host the '*Ca*. P. solani' in or around infected vineyards and can therefore play a role in BN spreading (Marchi et al., 2015; Mori et al., 2015; Oliveri et al., 2015). Molecular epidemiology approaches, using *vmp1*- and *stamp*-based markers allowed knowledge to be increased of the populations of BN throughout vineyards and their surroundings in the Mediterranean area (Fialová et al., 2009; Fabre et al., 2011; Foissac et al., 2013; Murolo et al., 2014; Landi et al., 2015; Murolo & Romanazzi, 2015; Pierro et al., 2018a, 2018b).

The complexity of BN disease epidemiology renders it difficult to design efficient control strategies. Insecticides applied to the grapevine canopy influence neither the disease nor the presence of *H. obsoletus* (Maixner, 2007; Mori et al., 2008b). The management of *H. obsoletus* host plants in the vineyards and surrounding areas is therefore considered crucial for BN control (Maixner, 2010; Mori et al., 2012; Panassiti et al., 2017). Thus, preventive measures, such as checking the health status of propagation materials (i.e., mother plants and grafted cuttings) and treating of cuttings through thermotherapy, are applied to limit long distance dissemination and in-field spread of the disease (Mannini et al., 2007). Other strategies for reducing BN spread or incidence are based on (i) preventive removal of the grape suckers on which *H. obsoletus* could feed after grass mowing (Picciau et al., 2010); (ii) trunk cutting above the engagement point on symptomatic grapevines (Kast et al., 2008; Riedle-Bauer et al., 2010); (iii) treatments by resistance inducers (Romanazzi et al., 2009, 2013). In prospective, also plant volatiles from host plants can be used for reducing vineyard colonization by *H. obsoletus* (Riolo *et al*., 2017).

In Israel, chaste tree (*Vitex agnus-castus* L.) is a plant where *H. obsoletus* can complete its life cycle (Sharon et al., 2005). In both olfactometric and field studies chaste tree resulted more attractive than grapevine for *H. obsoletus* adults (Sharon et al., 2005; Zahavi et al., 2007;~~,~~ Riolo et al., 2012). Therefore, a "push and pull" strategy based on the use of chaste tree as trap plant at vineyard borders to reduce the vector population living inside the vineyards was suggested (Zahavi et al., 2007). The validity of this strategy is reinforced by the fact that in Israel chaste tree was never found ~~to be~~ infected by '*Ca*. P. solani' and thus cannot serve as an inoculation source for grapevine (Sharon et al., 2015).~~ However, a study conducted in east Adriatic coast of Montenegro Eastern Europe reported the direct epidemiological role of *V. agnus-castus* as '*Ca*. P. solani' source in the *H. obsoletus*-mediated transmission to grapevine (Kosovac et al., 2016)~~

~~Considering such contradictory data, t~~This study aimed to investigate the possible role of ~~*Vitex*~~ *V.* *agnus-castus* as host plant of *H. obsoletus* and ~~'*Ca.* P. solani'~~CaPsol in n~~N~~orthern Italy. In detail, the interaction between chaste tree and *H. obsoletus* was examined through survival, attractiveness and oviposition trials, while the capability of chaste tree to harbor CaPsol ~~'*Ca.* P. solani'~~ in n~~N~~orthern Italy was studied through transmission trials in controlled conditions. In these studies, *H. obsoletus* adults collected on stinging nettle were used because this plant is the most important external source of infected vectors for Northern Italian vineyards (Mori et al., 2008b, 2015) and therefore the possibility of using chaste tree as trap plant at vineyard borders must be evaluated on this population.

2 MATERIAL AND METHODS

2.1 Survival of *Hyalesthes obsoletus* from stinging nettle on chaste tree and grapevine

Hyalesthes obsoletus adults were collected by using a sweep net and pooter in Veneto region on 4th July 2016 and 27th June 2017 from stinging nettle plants, growing along a ditch bordering a BN infected vineyard (45°23'32.42''N; 11°09'45.62''E), and were maintained for ten days under controlled conditions [25±3 °C, 70±5 RH, 16:8 (L:D) daily light cycle] in insects proof cages on potted plants of chaste tree, stinging nettle and grapevine~~ (cv Chardonnay)~~. The chaste tree plants were generated by tissue culture in Guagno nursery (Padova, Italy), stinging nettle plants were taken from field, and grapevine plants were one-year Chardonnay grafted on SO4 rootstock in Vivai Cooperativi Rauscedo (Pordenone, Italy). The plants, grown in 5 L pots, were in good vegetative condition and did not show any symptoms related to biotic and abiotic stresses. The three plant species had similar volume and leaf density (diameter about 0.3 m and high about 0.8 m).

Both years, the *H. obsoletus* individuals, collected from stinging nettle, were randomly confined on 8 singularly caged potted plants per each of the three host species. On average, in 2016 were used 28.5, 14.1, and 25.1 adults on chaste tree, stinging nettle and grapevine, respectively; in 2017, were used 14.6, 15.3, and 17.6 adults on chaste tree, stinging nettle and grapevine, respectively. Species recognition was confirmed based on the taxonomic keys by Bertin et al. (2010).

During the 10-day confinement, the number of dead individuals was counted daily. On the last sampling day the number of alive individuals was also counted, to know the total number in each cage. Kaplan-Meier analysis was used to estimate the survival curve on the three plants and the comparison between two survival curves was made by the log-rank test.

2.2 Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle

The attractiveness of chaste tree for *H. obsoletus* collected on stinging nettle was evaluated under laboratory, semi-field and field conditions.

(i) Laboratory conditions: the experiment was conducted in 2017 ~~on 40~~using *H. obsoletus* adults ~~(20 females and 20 males),~~ captured on stinging nettle (see survival trials §). Before their use in the experiment the ~~*H. obsoletus*~~ adults were left on Petri dishes with water for 12 hours. The planthoppers then underwent a choice test using a custom made two-choice olfactometer ~~(~~[following Dicke et al.~~,~~ (1988)~~)~~] between shoots of chaste tree vs grapevine (cv Chardonnay), chaste tree vs stinging nettle, stinging nettle vs grapevine~~chaste tree~~. The shoots were in good vegetative condition and did not show any symptoms related to biotic and abiotic stresses. Forty individuals (20 females and 20 males) were tested for each comparison. If 10 minutes after positioning the insect was still at the start on the olfactometer, the test was considered as "No choice". Data analysis was performed on the individuals that chose one of the two plants under comparison. To establish if the proportion of males and females that were attracted by one of the two plants was different, a Fisher's exact test was used. To know if one plant was ~~chosen by~~preferred by adults ~~adults (males plus females)~~ more than the other ~~under~~ in comparison, a G-test of goodness of fit was used. Since the percentages of males and females who have chosen one of the two plants under comparison are always differed for no more than 7%, this last analysis was conducted pulling together the adults of the two sexes.

(ii) Semi-field conditions: in 2016 and 2017, 9 cages (0.5 m × 0.5 m × 1.0 m) containing potted plants of two species~~20 *H. obsoletus* adults (10 females and 10 males) (captured on stinging nettle, see survival trials §) were confined in cages (0.5 m × 0.5 m × 1.0 m) containing potted plants of two species~~, namely chaste tree and grapevine (n. 3 cages) or chaste tree and nettle (n. 3 cages) or nettle and ~~chaste tree~~grapevine (n. 3) were prepared. The origin and the vegetative status of the plants were the same of those used in the survival experiment (see 2.1 §). The plants of the two species under comparison inside each cage were pruned to similar volume and leaf density (diameter of about 0.3 m and high of about 0.5 m). In each cage 20 *H. obsoletus* adults (10 females and 10 males) (captured on stinging nettle, see survival trials §) were confined~~.~~. Cages were maintained under controlled conditions [25±3 °C, 70±5 RH, 16:8 (L:D) daily light cycle]. Observation of adult insect's position was done 1, 4 and 8 hours after caging. If the insect was on the net or on the bottom of the cage, the position was considered as "No choice". ~~Three cages for each pair and for each year were used.~~ Data analysis was performed on the individuals that chose one of the two plants under comparison using a paired-sample *t* test.

(iii) Field conditions: the trial was conducted in 2017 in a ploughed field (3.7 ha surface, (45°23'34.92''N; 11°09'39.10''E) with one side (103 m long) bordered by a ditch covered with stinging nettle harbouring large *H. obsoletus* populations. At the time of the adults' flight period~~.~~ the stinging nettle along the ditch was mowed. Potted chaste tree, grapevine ~~(see survival trials §)~~ and nettle plants ~~(taken from nature and then potted in 5L pot)~~ (see survival trials §) were placed in the field at 5, 10 and 20 m from the border in the same day of the stinging nettle mowing. For each distance, 6 groups of the 3 plants were considered, one for each of the three species. T~~t~~he distance between each plant group was 15 m and 1.0 m between each plant within the group. All potted plants of the three species under comparison were pruned to similar volume and leaf density (diameter of about 0.4 m and high of about 0.9 m) and irrigated twice a week. The plants were in good vegetative condition and did not show any symptoms related to biotic and abiotic stresses. ~~Coinciding with the plants positioning, the stinging nettle along the ditch was mowed.~~ The presence of *H. obsoletus* adults on the three potted-plant species was monitored after nettle mowing by transparent sticky traps (A5 paper size 148 ×~~x~~ 210 mm) positioned within their canopy. The number of individuals captured during the first and second week was counted. To compare field-trial data (number of *H. obsoletus* adults captured), a three-way ANOVA was used, considering as source of variation sampling time (first and second week from stinging nettle mowing), host plant (stinging nettle, grapevine and chaste tree) and distance from *H. obsoletus* source (5 m, 10 m and 20 m). Prior to analysis data normality was tested with the Shapiro–Wilk test, homogeneity was tested with Levene's variance test, the presence of outliers was assessed, and the data were log(x+1) transformed. For *post hoc* comparisons of means, LSD5% (least significant difference between two means at the 5% level) was used.

2.3 Egg laying of *Hyalesthes obsoletus* from stinging nettle on chaste tree

Insects proof cages (0.5 m × 0.5 m × 1.0 m) were arranged on potted plants of chaste tree (grown from tissue culture Guagno nurseries – Padova) and stinging nettle (taken from nature). Four and eight potted plants for each species were considered in 2016 and 2017 respectively. The pots had 50 L of capacity and the holes at the bottom were closed with insect-proof net to allow water flow but prevent the hatched-nymphs escaping, The plants size was about 0.4 m in diameter and about 0.9 m in height.

In each cage 100 *H. obsoletus* adults (50 females and 50 males), collected on stinging nettle (see survival trials §) on 21st July 2016 and 14th July 2017, were confined with the plants. The cages were maintained in an open field during winter. In February 2017 and 2018, *H. obsoletus* nymphs were extracted from the soil by Berlese funnel and analysed under stereomicroscope. Nymphs were identified using the dichotomous keys of Cargnus et al. (2012). Data collected in the two years were analysed together using a paired-sample *t* test.

2.4. Transmission trials of BN phytoplasmas to chaste tree

In 2017 adults of *H. obsoletus* were collected on stinging nettle in a ditch bordering two BN-affected vineyards in Lombardy (Brescia province: 45°35’37.72’’N; 10°09’33.36’’E) and Veneto (Verona province: 45°23’32.42’’N; 11°09’45.62’’E) regions.~~ Species recognition was confirmed based on the taxonomic keys by Bertin et al. (2010).~~ Capturing of adults was done by using a sweep net and pooter. The captured insects were kept in jars for transport to the laboratory~~.~~. The transmission trials were conducted with twenty four chaste tree plants, tested PCR-negative for '*Ca.* P. solani' in a greenhouse under controlled conditions (25±3 °C, 70±5 RH) located in Verona province (45°20’13.72’’N; 11°13’03.28’’E). The plants were singularly caged and divided into three groups: (i) plants TBS1-TBS8, with confined *H. obsoletus* individuals collected in Brescia (30 adults per plant), (ii) plants TVR1-TVR8, with confined *H. obsoletus* individuals collected in Verona (30 adults per plant), and (iii) plants T1-T8, without insects (control plants). Transmission trials were left till the end of adult survival. After this period, the plants were kept in an insect-free greenhouse.

Dead insects (~~127~~ 136 in plants TBS1-TBS8; 146 in plants TVR1-TVR8), collected from the end of June till mid-July 2017, were stored in absolute ethanol at 4 °C. ‘*Ca*. P. solani’ was detected by nested PCR-based amplification of *stamp* gene (Fabre et al., 2011) using as templates the total nucleic acids extracted from both the individual insect specimens (Marzachì et al., 1998) and the leaves of chaste tree plants (Angelini et al., 2001) collected in October 2017 and 2018. The plants were kept in an insect-free greenhouse for the whole transmission period. Amplification products were analyz~~s~~ed by electrophoreses in 1% agarose gel stained with Midori green under a UV transilluminator.

PCR products (StampF1/StampR1), amplified from insect adults and chaste tree samples, were sequenced in both strands (Sanger method, 5X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pair in the software BioEdit, version 7.2.6 (Hall, 1999). Obtained *stamp* gene nucleotide sequences were aligned using the ClustalW Multiple Alignment program in the software BioEdit and analysed by Sequence Identity Matrix to estimate their genetic diversity. *Stamp* sequence variants, identified in the study, were aligned and compared with representative sequences of previously defined sequence variants (Pierro et al., 2018a, 2018b); a nucleotide sequence identity of 100% was necessary for the attribution to such sequence variants.

3 RESULTS

3.1 Survival of *Hyalesthes obsoletus* from stinging nettle on chaste tree and grapevine

The data gathered in the two years showed that adults of *H. obsoletus* collected from stinging nettle can survive on chaste tree and grapevine for some days, but the survival curves were significantly worse than those on stinging nettle (2016: grapevine vs stinging nettle, $X^2 = 251.4$, $p > 0.0001$; chaste tree vs stinging nettle, $X^2 = 104.6$, $p < 0.0001$; 2017: grapevine vs stinging nettle, $X^2 = 151.2$, $p > 0.0001$; chaste tree vs stinging nettle, $X^2 = 66.6$, $p < 0.0001$) (Figure 1). Survival on chaste tree and grapevine fell below 50% after three days in 2016 and after 5 days in 2017. In 2016 the survival curve on chaste tree was significantly better than on grapevine ($X^2 = 4.87$, $p = 0.016$), but in 2017 this difference was not confirmed ($X^2 = 1.47$, $p = 0.16$).

3.2 Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle

In the laboratory experiment with two-choice olfactometer, the proportion of males and females that chose one of the two plants under comparison with "no-choice" individuals was not significantly different ($p = 0.10$ for grapevine vs chaste tree, $p = 1$ for chaste tree vs stinging nettle, $p = 1$ for grapevine vs stinging nettle, Fisher's Exact Test). *Hyalesthes obsoletus* adults did not show any significant preference for grapevine vs chaste tree ($G = 0.081$, $p = 0.78$), chaste tree vs stinging nettle ($G = 0.081$, $p = 0.78$) or grapevine vs stinging nettle ($G = 2.19$, $p = 0.14$) (Figure 2).

In the semi-field experiment, there were significant differences in the choice of plant species by *H. obsoletus* adults collected on stinging nettle (Figure 3). In particular, chaste tree was significantly preferred to grapevine in both 2016 ($t = 2.80$, d.f. = 8, $p = 0.02$) and 2017 ($t = 2.80$, d.f. = 8, $p = 0.02$); stinging nettle was significantly preferred to grapevine in both 2016 ($t = 3.39$, d.f. = 8, $p = 0.0095$) and 2017 ($t = 5.58$, d.f. = 8, $p = 0.0005$); stinging nettle was significantly preferred to chaste tree in 2017 ($t = 2.44$, df = 8, $p = 0.04$), but not in 2016 ($t = 1.42$, d.f. = 8, $p = 0.19$).

In the open field, captures of *H. obsoletus* from stinging nettle plants along a ditch were significantly influenced by time (i.e., days from nettle mowing), plants and distance from *H. obsoletus* adults' source (Table 1). In particular, captures were higher the second than the first week from nettle mowing. On stinging nettle the captures were significantly higher than on the other two plants (Table 2). Although no individual was captured on grapevine, the differences with respect to chaste tree were not statistically significant based on LSD5% (Table 2). The captures decreased with the increase of distance from the ditch, i.e. from the source of *H. obsoletus* adults, and were significantly higher at 5 m than both 10 m and 20 m (Table 2). The interactions time × plant, plant × distance and time × plant × distance were significant due to the fact that the captures were influenced by time and distance only for stinging nettle and chaste tree, because on grapevines the captures were always zero (Table 1).

3.3 Egg laying of *Hyalesthes obsoletus* from stinging nettle on chaste tree

Based on the nymphs observed in February of the next year, *H. obsoletus* females laid eggs on potted plants in 10 out of 12 cages. Nymphs were recorded on the roots of both stinging nettle and chaste tree, showing indirectly that females had laid eggs on both plants, but a significantly higher number was observed on the former ($t = 3.36$, d.f. = 9; $p = 0.009$) (Figure 4).

3.4 Transmission trials

The PCR analyses for amplification of the *stamp* gene, performed on the total nucleic acids extracted from the chaste tree plants used in the transmission trials, showed the presence of '*Ca*. P. solani' in two plants (TBS6 and TBS7) out of 16 (12.5%). No amplification was observed in the other 14 chaste tree plants, on which insects were maintained, and on the eight control plants (without insects) (Table 3). The molecular analyses performed on the insect individuals collected from plants TBS6 and TBS7 revealed that five individuals out of 16 (31.25%) and six out of 18 (33.33%), respectively, were found to be infected by '*Ca*. P. solani'. *H. obsoletus* adults, collected from the 14 chaste tree plants negative to phytoplasma presence, were found to be infected at a percentage varying from ~~7~~ 0 to 50% (Table 3). Nucleotide sequence analyses of the *stamp* gene showed that chaste tree plants and insect individuals feeding on them harboured the same '*Ca*. P. solani' strain, characterized by the *stamp* gene sequence variant St5.

The PCR analyses performed on chaste tree leaves collected in October 2018 (one year after the transmission trials), showed that all 24 chaste tree plants, including TBS6 and TBS7 (positive in 2017), were negative to phytoplasma presence (Table 3).

DISCUSSION

Survival of *H. obsoletus* adults from stinging nettle was better on the plants on which the nymphs developed (i.e. stinging nettle), than on the other plants (i.e. grapevine and chaste tree). This occurrence was previously observed for *H. obsoletus* from stinging nettle or bindweed that had better survival on the origin plant than on the other (Mori et al., 2008; Kessler et al., 2011; Maixner et al., 2014). Survival on chaste tree was significantly better than on grapevine in one of the two study years. However, the differences were not so high as could be expected from the fact that chaste tree, unlike grapevine, is a true host of the planthopper (Sharon et al., 2015). Our study also indirectly confirmed that *H. obsoletus* can complete its life cycle on chaste tree because nymphs were observed in February on the roots of potted chaste tree plants on which planthopper adults had been caged and been able to lay eggs in the previous summer.

In the field, *H. obsoletus* adults from stinging nettle were more attracted by ~~potted plants of~~ stinging nettle than ~~either grapevine or~~ chaste tree ~~. Considering the two latter, as~~ and even not captureds ~~occurred in~~on grapevine~~, chaste tree seemed to be preferred~~. ~~The higher attractiveness of chaste tree compared to grapevine was showed by s~~Semi-field experiments confirmed both the scarce attractiveness of grapevine and the preference for stinging nettle than chaste tree~~in which even chaste tree was significantly less attractive than stinging nettle in only one of the two years~~. With reference to the two true host plants, ~~i.e.~~namely stinging nettle and chaste tree, preference for the former may ~~still~~ be associated with the origin of adults used for the experiments, ~~.~~all collected from stinging nettle plants. Based on this result, even the higher attractiveness of chaste tree in comparison with other plants observed in the olfactometer studies by Sharon et al. (2005) may have been influenced by the fact that most of the adults had been collected on chaste tree. The fact that chaste tree resulted significantly more attractive than grapevine would suggest its use as trap plant at vineyard borders. However, since the infected *H. obsoletus* adults that colonize vineyards in n~~N~~orthern Italy move mostly from stinging nettle and for this planthopper population the nettle was more attractive than chaste tree, the use of healthy potted plants of stinging nettle as trap plants would be preferable. Our two-choice olfactometric studies showed no significant preference by *H. obsoletus* for either of the two plants, even if fewer adults were observed on grapevine than stinging nettle.

Results of the transmission trials conducted in the study proved that chaste tree can harbour ‘*Ca*. P. solani’ and that infectious *H. obsoletus* adults from stinging nettle can inoculate this phytoplasma in chaste tree. This evidence is in agreement with the results obtained by Kosovac et al. (2016), who demonstrated that chaste tree naturally occurring in vineyard agro-ecosystems in Montenegro ~~Serbia~~ is infected by ‘*Ca*. P. solani’. The '*Ca*. P. solani' strain St5, transmitted with *H. obsoletus* originating from stinging nettle to chaste tree in the present study, is so far known to be associated only with bindweed as source plant, *H. obsoletus* from bindweed as vector, and grapevine in wide geographic European areas (Pierro et al., 2018). Moreover, strain St5 groups within the bindweed-related *stamp* phylogenetic Cluster b-II along with strains St1, St2, and St30, previously found associated with chaste tree or transmitted to grapevine by chaste tree associated *H. obsoletus* (Kosovac et al., 2016). Thus, this is the first report of strain St5 transmitted to chaste tree by *H. obsoletus* from stinging nettle. ~~Moreover, a~~As chaste tree constitutes an important reservoir for *H. obsoletus*-mediated transmission of BN phytoplasma to grapevine (Kosovac et al., 2016), our findings that chaste tree can host the '*Ca*. P. solani' strain St5, largely prevalent in the Franciacorta area, open a new intriguing scenario on its possible role in BN epidemiology in north Italy. On the contrary, these results are in disagreement with Sharon et al. (2005, 2015), who showed that, even if it is a preferred host plant of *H. obsoletus*, chaste tree did not harbour ‘*Ca.* P. solani’. Interestingly, even if '*Ca*. P. solani'-infected insect individuals were found on 15 out of 16 chaste tree plants used in transmission trials, *H. obsoletus* was only able to transmit the pathogen in two cases. This could be explained considering the short survival of insect adults on chaste tree; in fact, the insect populations decreased dramatically in 4 to 6 days after release. However, adults of *H. obsoletus* from stinging nettle survive on grapevine no better than on chaste tree and still are able to inoculate the BN phytoplasma. Moreover, the success of transmission trials can depend on the phytoplasma strain and its titer within the insect adults. For example, it is reasonable to hypothesize that '*Ca*. P. solani' strains not transmitted to chaste tree in the present study could be (as expected) those that are strictly associated with stinging nettle (*stamp* clusters a1 and a2). The fact that chaste tree plants, found positive for phytoplasma presence in October 2017, were phytoplasma-free in October 2018 can be explained by natural recovery from infection, as reported for a broad range of polyannual plants infected by phytoplasmas (Osler et al., 1993; Romanazzi et al., 2009), increased by abiotic stresses due to the overgrowth of chaste trees in pots under controlled conditions, which is not convenient in terms of spacing.

According to Sharon et al. (2005, 2015), showing that chaste tree is a preferred host plant of *H. obsoletus* and does not harbour '*Ca.* P. solani', in Israel a 'push & pull' strategy was suggested to reduce the population of *H. obsoletus* in a vineyard by using chaste tree as a trap plant (Zahavi et al., 2007). On the contrary, based on the findings of this and previous research work (Kosovac et al., 2016), it is doubtful that chaste tree can be used in the containment of the BN spread in Europe by using it as an attractant to *H. obsoletus* since it can also act as a reservoir of '*Ca.* P. solani'. However, volatiles from both chaste tree and stinging nettle could be used in the context of 'push & pull strategies' (Riolo et al., 2017).

In conclusion, the results obtained increased the knowledge about the role of ~~*Vitex*~~ *V. agnus-castus* as host plant of *H. obsoletus* and '*Candidatus* Phytoplasma solani' in n~~N~~orth Italy. Further studies are needed to determine the actual role of chaste tree in the BN epidemiology.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interests.

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