Action A2.
Preparation of protocols for the detection, identification and determination of pathogenicity assessment of alien fungi associated to Xylosandrus compactus and X. crassiusculus

Fast routine protocol to test pathogenicity of alien fungal species

Due date: 30/06/2019

Delivery date: 28/10/2019

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With support of: INRA-Biogeco

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INRA SCIENCE & IMPACT

Revision history

Version n°	Date	By whom	Concerns
1	20/09/2019	Carmen Morales-Rodriguez	Sent to the rest of A2 members
2	15/10/2019	Cécile Robin	Comments from INRA
3	28/10/2019	Carmen Morales-Rodriguez	Final editing
Final	28/10/2019	Andrea Vannini	Approved

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Summary

Symbiotic fungi associated to Ambrosia beetles contribute with insect damage to the impact to invaded environments. In addition of providing food for the insect development stages, some of the Ambrosia beetle symbiotic fungi are severe pathogens of plant hosts. The most relevant example is *Xyleborus glabratus* carrying the fungus *Raffaela lauricola* cause of a lethal vascular wilt of avocado, that is devastating the plantations in South-eastern United States. Thus, monitoring of alien fungi introduced through specific pathways (mostly trading of living plants), their identification and determination of pathogenicity behaviour is essential in order to design and apply prevention and mitigation quarantine measures. This deliverable describes the inoculation protocol of fungi associated to *X. compactus* and *X. crassiusculus*.

1. Two invasive species: *Xylosandrus compactus* and *X. crassiusculus*

1.1 *Xylosandrus compactus*

Xylosandrus compactus (black twig borer or shot-hole borer) it is a highly polyphagous pest of woody plants that probably originates from Asia and has been introduced to other parts of the world, most probably with trade of plants and wood. It is widely distributed in Africa, Asia and South America. It has been introduced in the Pacific Islands, New Zealand, Southeastern USA, and more recently in Europe in Italy and Southern France (EPPO Alert-list, Rabaglia et al., 2006, Wood, 1982; Chong et al., 2009). It was first found in Europe in 2011 (Garonna et al., 2012) in urban parks of the Campania region of Italy. Then, the species has been recorded in Italy's Campania, Tuscany and Liguria, and recently emerged in South-east France. The first report in Europe of *X. compactus* and associated ambrosia fungi in a natural environment has been recorded in September 2016, in the Italian National Park Circeo, Central Italy, in the Latium Region (Vannini et.al., 2016).

1.2. *Xylosandrus crassiusculus*

Xylosandrus crassiusculus (Asian ambrosia beetle or granulate ambrosia beetle) it is a highly polyphagous pest of woody plants of Asian origine and has been spread most probably with trade of plants and wood. In Africa it arrived hundreds of years ago while in the last few years it has been introduced to at least 14 African countries, 25 USA states, 3 countries of Central America, 2 South American countries, 6 countries of Oceania and 2 European countries (EPPO 2015, Fletchmann and Atkinson 2016), Italy and France, recently adding Spain (Gallego et al., 2017). Since the 1970s it has become a pest of fruit tree orchards and ornamental tree nurseries in the USA (EPPO Alert-list). It was first found in Europe in 2003, in cross-vane traps set up in Tuscany (Livorno, NW Italy) where no specific control measures were adopted (Pennacchio et al. 2003; EPPO 2015). Later, carob trees attacked by *X. crassiusculus* were found in in orchards in Central-North Italy and in gardens of nearby Liguria (Alassio and Pietraligure, NW Italy) in 2007 and 2008, and in NE Italy in Veneto (EPPO 2015) and Friuli Venezia Giulia (2015, personal

observation of Massimo Faccoli). Maybe via Liguria, in 2014 the species arrived in SE France and in the Spanish Valencia Region.

1.3. Invasiveness and impacts

X. compactus has been recorded from at least 224 tree species from 60 families (Greco et Wright, 2015). Besides the large range of dicotyledonous trees and shrubs, it attacks monocotyledonous plants (orchids and gingers) and conifers (Hara and Beardsley, 1979). It is included in the EPPO Alert List since February 2017 and CABI considers it a high-risk quarantine pest. It causes extremely serious infestations in domestic and natural environments, especially natural mixed-forest habitats. Females attack healthy twigs, boring into the living tissues and digging galleries in young branches of trees and shrubs (Ngoan et al., 1976).

X. crassiusculus is also a highly polyphagous species breeding in broadleaved trees in forests, plantations, and orchards. The beetles have been found on so many species, that it seems evident that almost any broad-leaved tree or sapling can be attacked, although the species has not been recorded from conifers. It is included in the EPPO Alert List (EPPO 2015) and CABI recommends its consideration as a high-risk quarantine pest.

The damage in plant species is related to both wood boring and the introduction of ambrosia fungi. Ambrosia fungi live associated with beetles in host trees and act as a food source for the insects. The symbiotic relation is important to the colonizing strategies of host trees by beetles. The fungi are highly specialized, adapted to a specific beetle and to the biotope where they both live. In addition, other fungi have been found such as tree pathogenic fungi that may play a role in insects host colonization success. Saprophytic fungi are also present in insect's galleries. These may decompose cellulose and/or be antagonistic to other less beneficial fungi (Henriquez et al., 2006).

2. Protocol to test pathogenicity of fungal species

In a first step isolate to test are grown in PDA media (cf appendix 1), 4 or 1 mm agar disk from colony edges will be used for the inoculation. The isolates are applied on the main stems of trees on wounds done following two methods: a circular wounding done at the cambium level (4 mm diameter, modality A) and at the xylem level (1 mm diameter, modality B). On these wounds, a

mycelial disk will be applied covered with the removed bark piece and autoclaved wet gauze, and sealed with Parafilm (Figure 1).

Figure 1. Different steps of inoculation methods; from left to right: Circular bark wounding, mycelial disk application and coverage with autoclaved wet gauze, Parafilm seal.

Symptom assessments of visual wilting (Figure 2) are done weekly the first two weeks. Fifteen days after inoculation, on half of the plants and 30 days the rest, external bark is removed to assess the internal lesion (Figure 2).

Figure 2. Inoculated *Quercus ilex* with *Fusarium solani* isolated from *X. compactus*.

To confirm the causative relationship between the inoculated fungi and damage observed Koch's postulates should be applied with the re-isolation of the fungi from the necrosis as described below.

3. Isolation of inoculated fungal species

Direct plating of end of the necroses occurred first by surface sterilizing the wood samples with 95% ethanol for 40 s, and then by placing 2-3mm bark samples removed with a sterile scalpel onto Potato Dextrose Agar (PDA) amended with streptomycin and ampicillin (6 pieces per plate).

Plates are incubated at 25°C for up to 2 wk. Morphotype designations are confirmed by comparing pure cultures and by DNA sequencing as described below in the case of the morphological aspect not be enough.

4. Fungal identification-DNA barcoding

Pure culture isolates are grown in PDA (potato dextrose agar) with a layer of cellophane, the mycelia is harvested and grinded with a ball mills. The genomic DNA is extracted from 100 mg of mycelium using a commercial DNA extraction kit (e.g. Nucleospin Plant II kit (Machery Nagel, Duren, Germany) or CTAB protocol (Appendix 2)).

The variable internal transcribed spacer 1 (ITS1F) is amplified. Each PCR contained 12.5 µl of 2X MyTaq Mix, 1 µl of each primer (10 µM, and 1 µl of genomic DNA in a total volume of 25 µl. The primer pairs for amplifying internal transcribed spacers are ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR consist of an initial denaturing step of 5 min at 94°C followed by 35 cycles (15 s at 94°C, 15 s at 55°C and 10 s at 72°C) finished by a final extension step at 72°C for 10 min.

PCR products are resolved by electrophoresis through 1.5% agarose gels in TBE and visualized by staining with Gel Red (Biotium). Amplicons are purified using the Nucleospin Gel and PCR Clean-up kit (Machery Nagel, Duren, Germany) and quantified with Qubit Quantification kit (Invitrogen, USA) and they are Sanger sequencing.

After the assembling of forward and reverse, the consensus sequence is blasted at the NCBI database.

In the case of the ITS region does not provide enough reliability for the identification of the fungal isolate other genetic markers have to be done as B-tubulin or Elongation factor.

B-tububulin: Primers T1/Btub-2b

Program: 5 min 94°C,

40x (45 sec 94°C, 30 sec 52°C, 90 sec 72°C),

6 min 72°C, quick cooling at 4°C

Elongation factor: Primers EF1-728F/EF-2

Program: 5 min 94°C,

40x (45 sec 94°C, 30 sec 52°C, 90 sec 72°C),

6 min 72°C, quick cooling to 4°C

Appendix 1: Media preparation

Media is sterilized by autoclaving at 121°C for 15 min unless stated otherwise by the supplier. Prepared plates should be stored at 2–8°C in the dark.

- **PDA (potato dextrose agar)**

PDA (potato extract 0.4%, dextrose 2%, microbiological grade agar 1.5%)	39.0 g
Distilled water to make up to pH = 5.6 ± 0.2	1 L

If the medium is to be used for galleries fungal isolation, after autoclaving and cooling at about 50°C, add a sterile water-diluted streptomycin and ampicilin aliquot, at a final concentration of 0.05 g L^{-1} and 0.25 respectively

- **PBS (Phosphate-buffered saline)**

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving. Store PBS at room temperature.

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentratio n (10×)
NaCl	8 g	137 mm	80 g	1.37 m
KCl	0.2 g	2.7 mm	2 g	27 mm
Na_2HPO_4	1.44 g	10 mm	14.4 g	100 mm
KH_2PO_4	0.24 g	1.8 mm	2.4 g	18 mm

Appendix 2: CTAB (cetyl trimethyl ammonium bromide) DNA extraction

Freeze dried mycelium is ground at room temperature (15 seconds at 30 Hz using a Retch mill and a metal ball per tube).

For each 50 mg homogenized mycelium, use 400 µl of CTAB Extraction Buffer. Mix and thoroughly vortex. Transfer the homogenate to a 65°C bath for 60 minutes and shake every 30 minutes.

Once the lysis is carried out, add 400 µl of chloroform isoamyl alcohol.

Put the samples at -20 ° C for about 2 hours. Following this,

Once the samples are taken out and thawed, centrifuge them for 15 minutes at 4 ° C at maximum speed.

Remove the supernatant by overturning the tubes being careful that the formed pellet does not come off.

Wash with ethanol at 70 °, 500 µL per tube, and centrifuge with the same conditions as the previous one, for only 10 minutes. The ethanol is removed and the tubes are evaporated until there is no more ethanol on the walls of the tube.

Finally, the pellet is resuspended in 100 µl of water and the DNA samples obtained are stored at -20 ° C.

CTAB buffer:

2%:CTAB (hexadecyltrimethylammonium bromide)

100 mM TrisHCl [pH=8]

20 mM EDTA,

1.4 M NaCl

0.2% β-mercaptoethanol [added just before use]

0.1 mg/mL proteinase K [added just before use])