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

Deliverable

Fast routine protocol for detection of fungal symbiotic community associated to trapped *Xylosandrus*

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Revision history

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1	05/04/2019	Carmen Morales-Rodriguez (UNITUS)	Revision of the early version of protocols for stakeholders information
2	20/05/2019	Carmen Morales-Rodriguez (UNITUS)	Sent to INRA for the review and comments
3	23/06/2019	Cecile Robin (INRA)	Comments to the protocol
4	24/06/2019	Marta VanLeijen srl	corrections
Final			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 detection, isolation and identification protocols of the fungal community 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).

The adult females are dark brown to almost shiny black, 1.4-1.9 mm long and about two times longer than wide. The small, wingless males are reddish black and measuring 0.9–1.3 mm in length (Hara & Beardsley, 1979). *Xylosandrus compactus* is an arrhenotokus species in which males are born from unfertilized eggs (0.3 -0.5 mm) and females from fertilized ones. After mating, which primarily occurs between siblings just after adult emergence, the male remains in the gallery while the female leaves the tunnel through the entry hole and colonizes branches of new hosts, boring an entry hole and a subsequent brood gallery (Hara & Beardsley, 1979; Greco & Wright, 2015). (CABI Factsheet).

1.2. *Xylosandrus crassiusculus*

Xylosandrus crassiusculus (Asian ambrosia beetle or granulate ambrosia beetle) it is a highly polyphagous pest of woody plants of Asian origin 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.

Adults are small dark reddish brown scolytids (female: 2-3 mm long, males: 1.5 mm). Larvae are white, legless, C-shaped with a well-developed capsule, and cannot be easily distinguished from other scolytids. Populations essentially contain females (1:10 male-female ratio). Adult males do not fly and remain inside the galleries. Also *X. crassiusculus* is an inbreeding species (females mate with their brothers).

When females emerge, they leave infested plants and fly to new hosts. They start to bore a tunnel (round entrance hole of 2 mm diameter) with a brood chamber and one or more branches into the sapwood (and sometimes the heartwood). Eggs are laid in the brood chamber. Larvae have a length about 3.5 mm. and hatch and feed on the symbiotic fungus growing inside the galleries (Gardner, 1934, CABI Factsheet).

The damage in vegetal 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. Fungal isolation protocol from insect's galleries

Direct plating of beetle galleries occurred first by surface sterilizing the wood samples with 95% ethanol for 40 seconds, and then by placing 2-3mm gallery 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 weeks. Morphotype designations are confirmed by comparing pure cultures and by DNA sequencing as described below.

3. Fungal isolation from insects

The beetles included in fungal isolations are collected in flight from ethanol-baited traps. One to five adult *Xyloxandrus* sp. females per sample are used for fungal isolation. Insects are stored in tubes at 5°C for a maximum of three days prior to fungus extraction.

The surface of each beetle is washed by vortexing in a 1 ml sterile solution of 1% Tween 80 (Sigma Chemical Co, St. Louis, MO) and phosphate buffer saline (PBS) and then is serially diluted prior to plating. Following the wash, beetles are vortexed for 15 seconds in 1 ml sterile PBS and allowed to dry on tissue paper.

The insects are crumbled into an Eppendorf tube with the help of a mortar with 200 microliters of PBS. Add 800 microliters more of PBS to arrive a 1 ml of total volume and vortex the sample.

Serial dilutions (1/10 and 1/100) are done from each sample (surface wash and insects). Five hundred microliters of each dilutions are plated on PDA (ten plate per dilution) and cultures are incubated at 25°C for up to 2 weeks. Different colonies are sub-cultured in new PDA plates. Morphotype designations are confirmed by DNA sequencing as described below.

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 sufficient 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

5. High throughput sequencing (HTS) protocol for symbiotic fungi detection from insects and plant tissues

5.1 DNA extraction and ITS1 amplification

Total DNA from an average of 5 adults per sample are extracted using the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's instructions. Total DNA from wood tissues from insect's galleries is extracted using the Nucleospin Plant II kit (Machery Nagel, Duren, Germany) according to the manufacturer's protocols.

The ITS1 region is amplified with a dual indexing primers using the tagged primer pair ITS1F (5'-xxxxCTYGGTCATTTAGAGGAAGTAA-3') and ITS2 (5-xxxxGCHRCGTTCTTCATCGDTGC-3'), where xxx represents the barcoding key. The PCR reaction mixture consisted in 12.5 µl of Maxima Hot Start PCR Master Mix (2X) (Thermo Fisher Scientific, USA) and 1 µM of each primer in a total volume of 25 µl containing 24 µl of reaction mixture and 1 µl of template. The thermal cycle is an initial denaturation at 94 °C for 10 min followed by 30 cycles of 95 °C for 40 s, 60 °C for 40 s and 72 °C for 1 min, and a final elongation at 72 °C for 10 min. Four PCR are done and pooled per sample.

Amplicons are purified using the MagJET NGS Cleanup (Thermo scientific, USA), quantified with the Qubit Quantitation kit (Invitrogen, USA). Paired-end sequencing (2 x 300 bp) is carried out on an Illumina MiSeq sequencer.

5.2 Bioinformatics analysis

To reduce the risk of cross-contamination and false assignments, only the reads containing the combination of 5'barcode and forward primer as well as the expected 3'barcode and reverse primer are paired and used in the analyzes moreover, for the identification of barcode and primer sequences no mismatches are allowed. Raw read pairs are quality filtered (limit=0.05) and trimmed using CLC Genomic Workbench Version 8.5.1 (QIAGEN Aarhus, Denmark) filtered out all sequences containing "N"s and sequences with minimum length of 100 nucleotides and maximum length of 400 nucleotides.

Following, the paired-end reads are assembled. If there are mismatches between the overlapping fragments of the forward and reverse reads, these are corrected according to the base call with the higher sequencer-assigned quality score.

After the quality filtering, paired-end assembly and demultiplexing the sequences are processed, and a similarity clustering is done based on the UPARSE pipeline of the USEARCH v8 (Edgar, 2003) with a 97% of clustering threshold (Lindahl et al., 2013). Sequences failing alignment or identified as chimeric are removed before downstream analysis.

The consensus OTUs are identified using the BLAST tool in the Genbank database with the algorithm parameters word size=11, match/mismatch scores=2,-3, gap cost existence =5 and gap cost extension=2. The .xml file from the BLAST and the blasted fasta file are imported into

MEGAN (Huson et al., 2011) to compute and explore the taxonomical content of the data set, employing the NCBI taxonomy to summarize and order the results. Lowest common ancestor parameters are: Min score=170; Max. expected=0.01; Top percent=2.0, Min support percent=0.3; Min support=1 and LCA percent=40) and with the following minimum requirements of similarity to accept the proposed taxonomy: *Species* 99%, *Genus* 97%, Family 95%, Order 90%, Class *85%*, and *Phylum 80%*.

Finally, the OTU abundance table are generated with USEARCH v8 (Edgar, 2013; Bálint et al., 2014). Any OTU representing less than 0.001% of the total filtered sequences are removed to avoid inclusion of erroneous reads, leading to inflated estimates of diversity (Parks et al., 2013).

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 concentration (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])