REDIA, XCVIII, 2015: 115-122

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MOLECULAR DIAGNOSIS OF *BURSAPHELENCHUS XYLOPHILUS* (STEINER & BUHRER) NICKLE (NEMATODA APHELENCHIDA PARASITAPHELENCHIDAE) IN PRESENCE OF POLYPHENOLS AND OTHER INHIBITORY COMPOUNDS ([1])

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Strangi A., Torrini G., Carletti B., Roversi P.F. – Molecular diagnosis of *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Nematoda Aphelenchida Parasitaphelenchidae) in presence of polyphenols and other inhibitory compounds.

The pine wood nematode *Bursaphelenchus xylophilus* (Aphelenchida Parasitaphelenchidae) infects conifers, in particular pines, causing pine wilt disease. This nematode is considered a quarantine organism according to the EPPO and AQISQ guidelines. For this reason, several procedures for an affordable diagnostic test have been developed based on LAMP technique. In this work we shown how this technique behaved in presence of compounds that exert an inhibitory effect on DNA polymerases, a common problem that could be found in DNA preparations obtained from samples included in plant matrixes

LAMP technique was found able to detect presence of its specific DNA target in almost all cases studied but we found that the addiction of polyphenolic compounds present in some woody matrixes to DNA preparations could affect significantly on the sensibility of diagnostic test, while resinous material had a lesser influence on amplification.

KEY WORDS: *Bursaphelenchus xylophilus*, quarantine nematodes, phytopathological inspection, diagnostic techniques.

INTRODUCTION

The pine wood nematode (PNW) *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Aphelenchida Parasitaphelenchidae) is a migratory endoparasitic nematode identified as the causal agent of pine wilt disease (WINGFIELD *et al.*, 1986). *B. xylophilus* is indigenous to North America and is widespread in natural coniferous forests in Canada and USA (WINGFIELD *et al.*, 1982a). On its natural hosts it behaves like other members of the genus, with a mycophagous life cycle on trees weakened or damaged by other causes. When introduced into non-native areas, it encounter new host species of *Pinus*, some of which are exceptionally susceptible so that the nematode follows a 'phytophagous' life cycle. The introduction and spread of this nematode has resulted in heavy wood losses in coniferous forest trees in East Asia, North America and Europe (EVANS *et al.*, 1996; MOTA *et al.*, 1999; ABELLEIRA *et al.*, 2011; FONSECA *et al.*, 2012). Outbreaks are reported in Japan, China, Korea, Taiwan, Spain and Portugal (WINGFIELD *et al.*, 1982a,b; TZEAN & JAN, 1985; YI *et al.*, 1989; MOTA *et al.*, 1999; BAOJUN *et al.*, 2001; KARNKOWSKI & SAHAJDAK, 2010). *B. xylophilus* is considered a quarantine organisms according to the EPPO and AQSIQ guidelines and it is included in USDA APHIS export regulations for mill products.

The most recent diagnostic tests use LAMP for detection of *B. xylophilus* DNA (KIKUCHI *et al.*, 2009; KANG *et al.*, 2014), while more refined protocols use retrotranscription of specific mRNA to distinguish between molecular traces from dead and alive specimens present in the sample (LEAL *et al.*, 2013, 2014). In view of the high number of analyses performed daily in phytosanitary and customs laboratories, first-screening diagnostic procedures should be as fast (and simple) as possible. Problematic samples could be hindrance, particularly if the diagnostic laboratory is not well equipped to perform more thorough analyses.

The presence of substances that inhibit DNA polymerases is a frequent problem for amplification systems used to detect plant pathogens of woody hosts (LANGRELL & BARBARA, 2001). Polysaccharides and secondary plant metabolites (such as lignin, polyphenols and various phenolic compounds) are well-known inhibitory contaminants of DNA preparations obtained from wood matrices (STRANGI *et al.*, 2012). Although several plant DNA extraction procedures and commercially-available kits have been designed to minimize co-extraction of inhibitory compounds, inhibition remains a major impediment to many routine applications, compromising both sensitivity and reliability of diagnostic essays (BICKLEY & HOPKINS, 1999). In phytosanitary laboratories, repetition of sampling may not be possible, especially for small, perishable or valuable goods (ornamental trees, bonsai, artistic artifacts, etc.). Therefore, guidelines for affordable analyses of these kinds of samples would be useful.

MATERIAL AND METHODS

DNA fragments of the ITS locus obtained from *B. xylophilus* strain BxPt7 were cloned in pCRII using the

[1] This work was supported by a grant from the Italian Ministry of Agriculture, Food and Forestry national project "IPPC-FAO-Pine Wood Nematode Risk" (DM 10909/7303/11).

– Received 30 September 2015 Accepted 3 November 2015 Published 10 December 2015

Dual Promoter TA Cloning kit (Invitrogen) as per the manufacturer's instructions. The products were screened for the ITS default restriction pattern (BURGERMEISTER *et al.*, 2005) and the selected clone was sequenced. The chosen construct was called *pCRII*[B.xyloITS] and was quantified with Qubit + HS dsDNA assay kit.

Samples of bark, wood and resin were collected from *Pinus pinea*, *Pinus pinaster*, *Pinus nigra*, *Abies alba*, *Picea abies*, *Cedrus atlantica* and *Cupressus sempervirens*. The plant tissues (2.0 mg each) were put in 50.0 µl of InstaGene Matrix (Bio-Rad), pestled with a micropipette tip and spun. Samples and controls were incubated in a thermocycler (2720 Thermo Cycler, Applied Biosystems) following the default protocol of InstaGene Matrix to obtain solutions containing an amount of polyphenols that might be co-extracted using a routine DNA extraction protocol. Solutions of inhibitors were characterized whit UV-VIS spectrophotometry measuring absorbance between 230 to 750 nm (QIAexpert, QIAGEN). Solutions containing polyphenols were serially diluted 2-, 5-, 10-, 20-, 50- fold; *pCRII*[B.xyloITS] was added at a final concentration of 1.5 ng μl^{-1} to maintain an equal concentration of template DNA in all assays obtaining artificially-contaminated plasmid preparations.

LAMP reactions were performed using the following protocol: reaction was performed in a total volume of 25.0 µl using 5.0 µl of artificially-contaminated plasmid preparation, primers developed by Kikuchi et al.(KIKUCHI *et al.*, 2009) at same concentration described in that study and 15.0 µl of Isothermal Master Mix (OptiGene) as per the manufacturer's instructions. Isothermal amplification was performed in a Genie III (OptiGene) thermal machine. The same serial dilutions of inhibitors without the addition of *pCRII*[B.xyloITS] were used as negative controls, while 6.5 ng of construct (final concentration 1.5 ng μl^{-1}) without the addition of inhibitors were used as a positive control.

To test minimum amplifiable DNA concentration, 1- to 50-folds serial dilutions of single nematode DNA preparation was tested. LAMP was performed using previously described protocol starting from 5.0 µl of DNA preparation, and 6.5 ng of construct were used as a positive control.

To assess if specificity of diagnostic test could be retained in our experimental conditions, single nematodes of the different *Bursaphelenchus* species [*Bursaphelenchus xylophilus* strains BxUS1 (USA) and BxPt7 (Portugal); *B. mucronatus* strains IT13 (Italy), IT1 (Russia), F2 (France), VR473 (Austria) and C60/11 (Portugal); *B. thailandae* strain RC-A (China); *B. fraudulentus* strain IT23 (Italy); *B. eremus* strains IT18 (Italy), IT19 (Italy) and DE39 (Germany)] were put in 50.0 µl of InstaGene Matrix (Biorad) and DNA was extracted according to manufacturer's protocol. ITS loci belonging to all *Bursaphelenchus* strains used in this work were amplified as in BURGERMEISTER *et al.* (2005) and subsequently submitted to GeneBank. LAMP was performed using previously described protocol starting from 5.0 µl of DNA preparation.

RESULTS AND DISCUSSION

Presence of different compounds and their relative concentrations in bark, wood and resin extracts was confirmed by comparison of absorbance profiles. The shape of the curves differs significantly as shown in Fig. I. More in detail, resin extracts showed a unique absorption peak centered about at 250 nm, while bark and wood extracts commonly showed multiple peaks clustered from 230 to 280 nm, in the lowest wavelength investigated. Comparing absorbance profiles obtained from plant extracts with absorption spectra of lignin solutions, it can be possible identify only in extracts of bark and wood a peak between 274 - 276 nm or 281 - 285 nm; those peaks were cosidered characteristics of different isoform of lignin, lignin from hard-wood and soft-wood respectively (STAMM *et al.,* 1932**).** Differences in absorbance profiles could be found also at genus level, such as, for example, when comparing *P. pinea* and *P. pinaster* (Fig. I, 1, 2) extracts with those from *P. nigra* (Fig. I, 3).

The addiction of *pCRII*[B.xyloITS] construct at a defined concentration in all extracts and dilutions allow us to obtain solutions where the only variable to test were the effect of inhibitors on LAMP amplification, in order to simulate contaminated samples. The LAMP technique was able to detect the *pCRII*[B.xyloITS] construct in almost all artificially contaminated samples as shown in Fig. II. The strongest inhibitory activity was shown by *A. alba* tissues (Fig. II, 4, 11, 18) and the weakest by *P. pinaster* (Fig. II, 2, 9, 16). In general, polyphenols extracted from bark tissues showed stronger (or in two cases equal) inhibition than those from the wood of the same plants. Samples mixed with *Pinus* bark polyphenols (Fig. II, 1, 2, 3) showed less inhibition of LAMP from 2-fold to 10-fold dilution than those coming from *P. abies* (Fig. II, 5) and *C. sempervirens* (Fig. II, 7), 50-fold dilution and 20-fold dilution respectively; no amplification was detected with *A. alba* bark at 50-fold dilution (Fig. II, 4).

Samples containing extracts from wood of *A. alba* and *P. abies* (Fig. II, 11, 12) showed amplification starting from 20-fold dilution, while *pCRII*[B.xyloITS] could be detected in samples containing *P. pinaster* and *C. sempervirens* extracts even when undiluted (Fig. II, 9, 14). Samples mixed with resins contained less inhibitors of the LAMP reaction than lignin-containing tissues (bark and wood). Amplification from undiluted samples was recorded for *P. pinea*, *P. nigra* and *C. atlantica* (Fig. II, 15, 17, 20); the highest dilution was 10-fold in *C. sempervirens* (Fig. II, 21).

Remarkably, serial dilution of bark and wood polyphenols influenced the height of the fluorescence plateau, which is directly related to the amount of amplification product generated by the LAMP reaction. This finding could be justified considering the inhibition mechanism of polyphenolic compounds; indeed it is known their ability to be oxidized into quinones and bind exposed sulfhydryl and amino groups of surface-exposed amino acid residues of proteins. This covalent binding lead enzymes to alterate their 3D structure and, consequently, loss their function. This inhibition mechanism is irreversible and lead to subtract DNA polymerase to amplification reaction, probably causing a decrease in the amount of amplification product (BITTNER, 2006; OSMAN *et al*, 2006; HE *et al.*, 2007).

Instead, samples mixed with serial dilutions of different resins generally showed the same fluorescence plateau but different reaction rates, with the only exception of *P. abies* resin. This difference could be explained by the presence of a different inhibition mechanism operated by compounds that could be found in resin rather than in lignified tissues.

Amplification of the *pCRII*[B.xyloITS] construct was detected in the absence of inhibitors in all LAMP reactions. No amplification was detected in plant tissue extracts in the absence of construct (data not shown).

Our results suggest that the use of very low amounts of template DNA could not affect sensibility nor specificity

of diagnostic test. In fact genomic DNA extracted from a single *B. xylophilus* nematode could be detected at all dilutions tested (Fig. III, 1). Furthermore, LAMP retained its specificity in the experimental conditions used with previously untested strains of *B. mucronatus*, *B. thailandae* and *B. fraudulentus*. Moreover, LAMP was able to discriminate between *B. xylophilus* and *B. eremus*, a previously untested species (Fig. III, 2, 3). Sequences of ITS loci belonging to *Bursaphelenchus* strains used in this work were submitted in GeneBank with accession numbers: KP644759, KP644760, KP644761, KP644762, KP644763, KP644764, KP644765, KP644766, KP644767, KP644768, KP644769 and KP644770.

LAMP technique showed a very good compromise between sensibility and resistance to inhibitor compounds that could be released in DNA preparations from PWN extracted from coniferous tissues without affecting specificity of diagnostic test. However, particular attention could be paid to polyphenols-containing samples rather than resin-containing ones, because presence of polyphenols strongly affects the amount of final amplification product and this, in turns, is directly linked with the formation of pyrophosphate that is the required substrate for the coupled reaction used in many commercial diagnostic kits that leads to detection of fluorescence. The effect of inhibition could be so important to prevent the detection of fluorescence with naked eye, and for this reason in this case a more accurate diagnostic procedure would be required. Moreover, it could be necessary a photometrical comparison between the fluorescence of sample with that recorded from a positive internal control that runs in parallel whit the diagnostic test.

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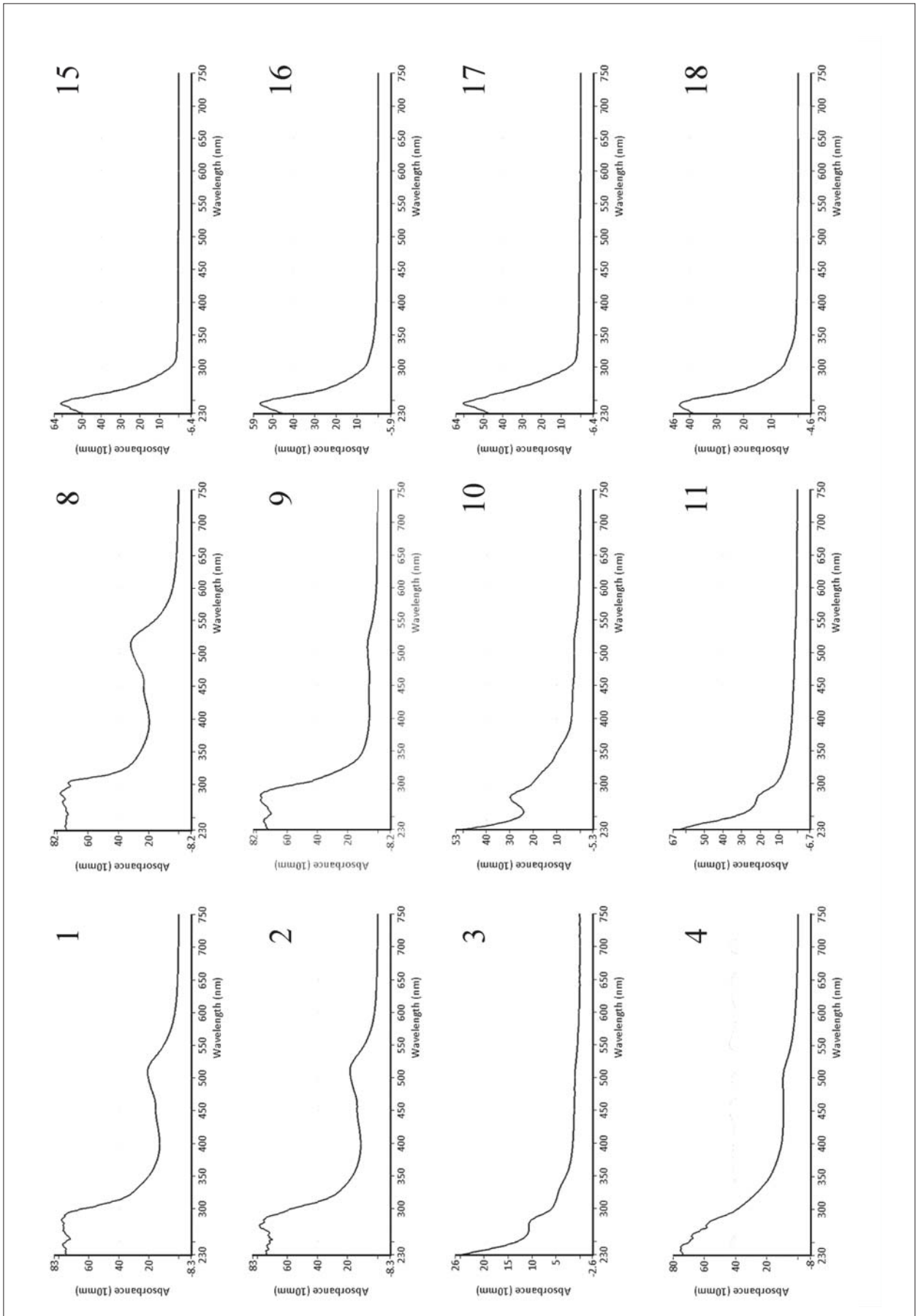
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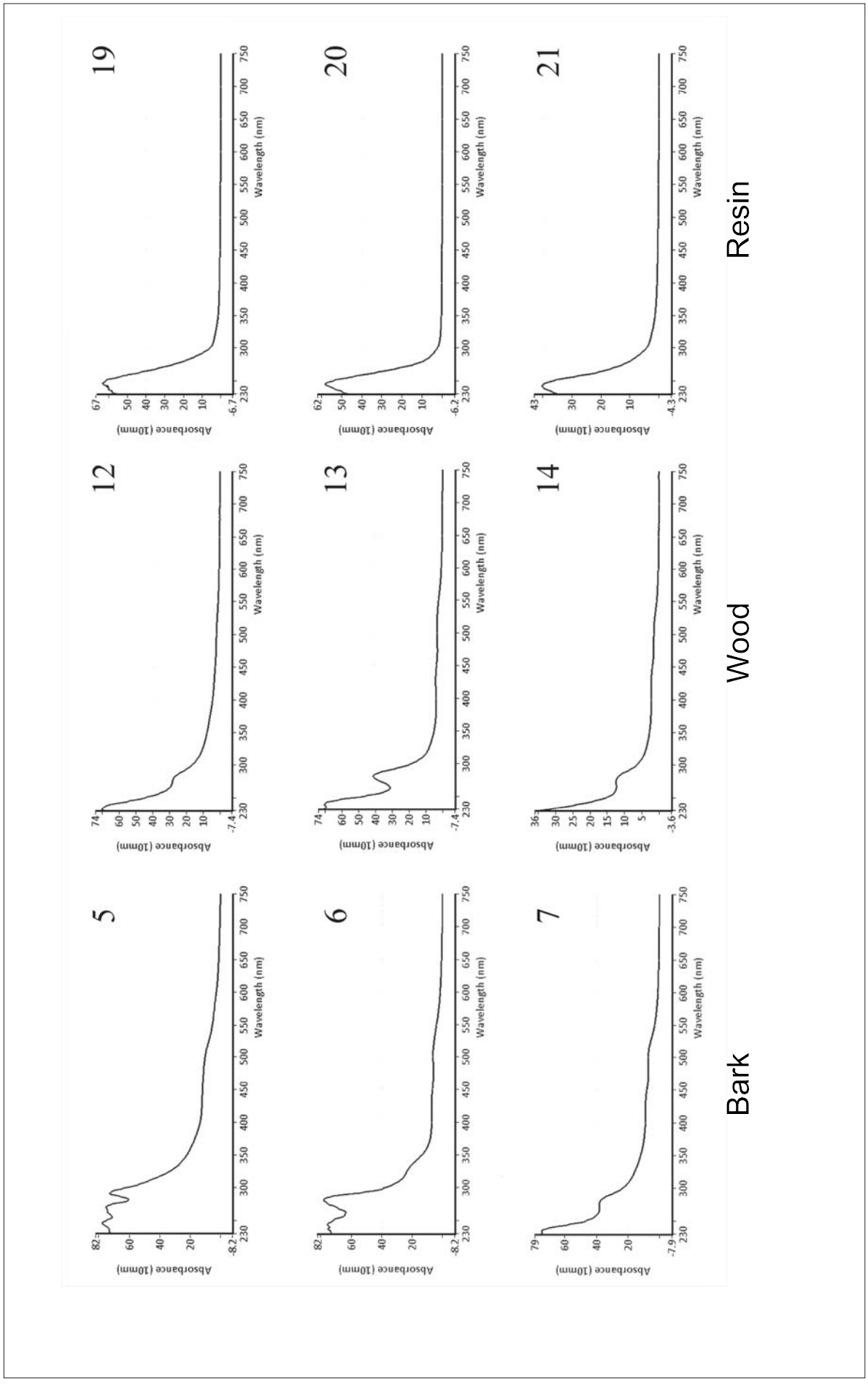
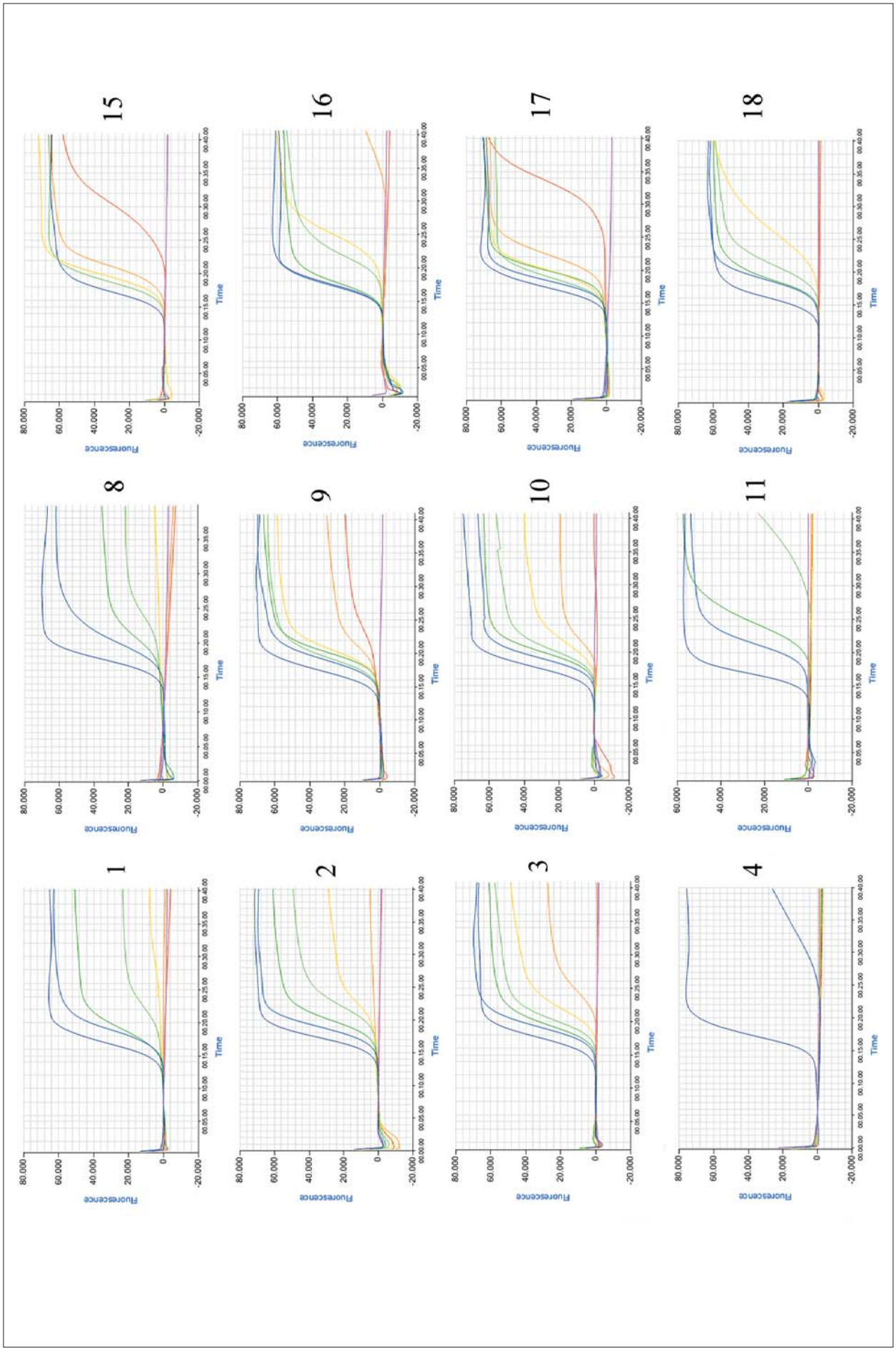


Fig. I – Absorbance profiles of extracts obtained from extracts of 1., 8. and 15. *Pinus pinea*; 2., 9. and 16. *P. pinaster;* 3., 10. and 17. *P. nigra*; 4., 11. and 18. *Abies alba*; 5., 12. and 19. *Picea abies*; 6., 13. and 20. *Cedrus atlantica*; 7., 14. and 21. *Cupressus sempervirens*.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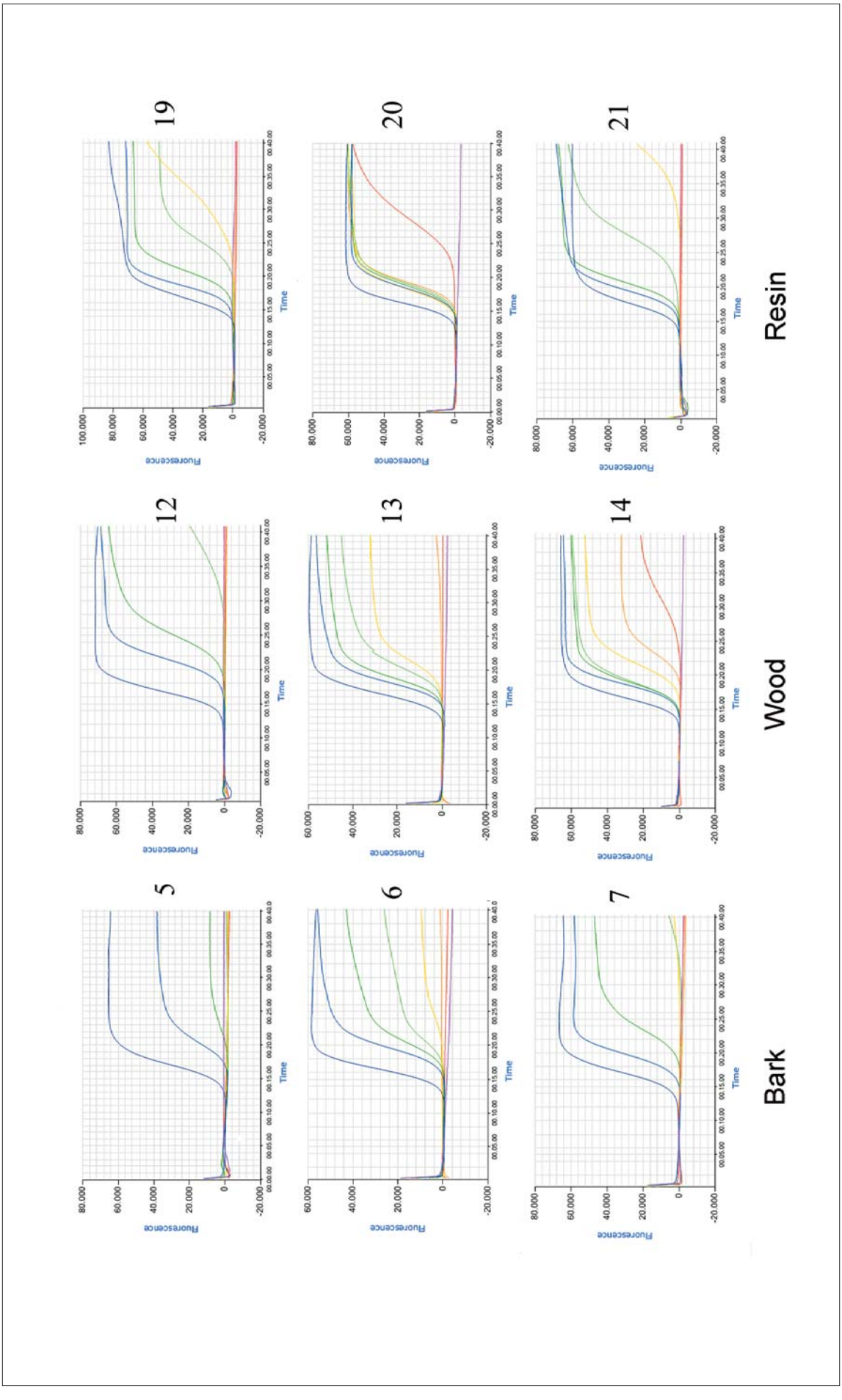

Fig. II – Detection of *pCRII*[B.xyloITS] in dilutions of artificially-contaminated samples with extracts of 1., 8. and 15. *Pinus pinea*; 2., 9. and 16. *P. pinaster*; 3., 10. and 17. *P. nigra*; 4., 11. and 18. *Abies alba*; 5., 12. and 19. *Picea abies*; 6., 13. and 20. *Cedrus atlantica*; 7., 14. and 21. *Cupressus sempervirens*. Colour legend: Red - undiluted extract, Orange - 2-fold dilution, Yellow – 5-fold dilution, Light green – 10-fold dilution, Dark green – 20-fold dilution, Blue – 50-fold dilution, Dark purple – Positive control, Violet – White.

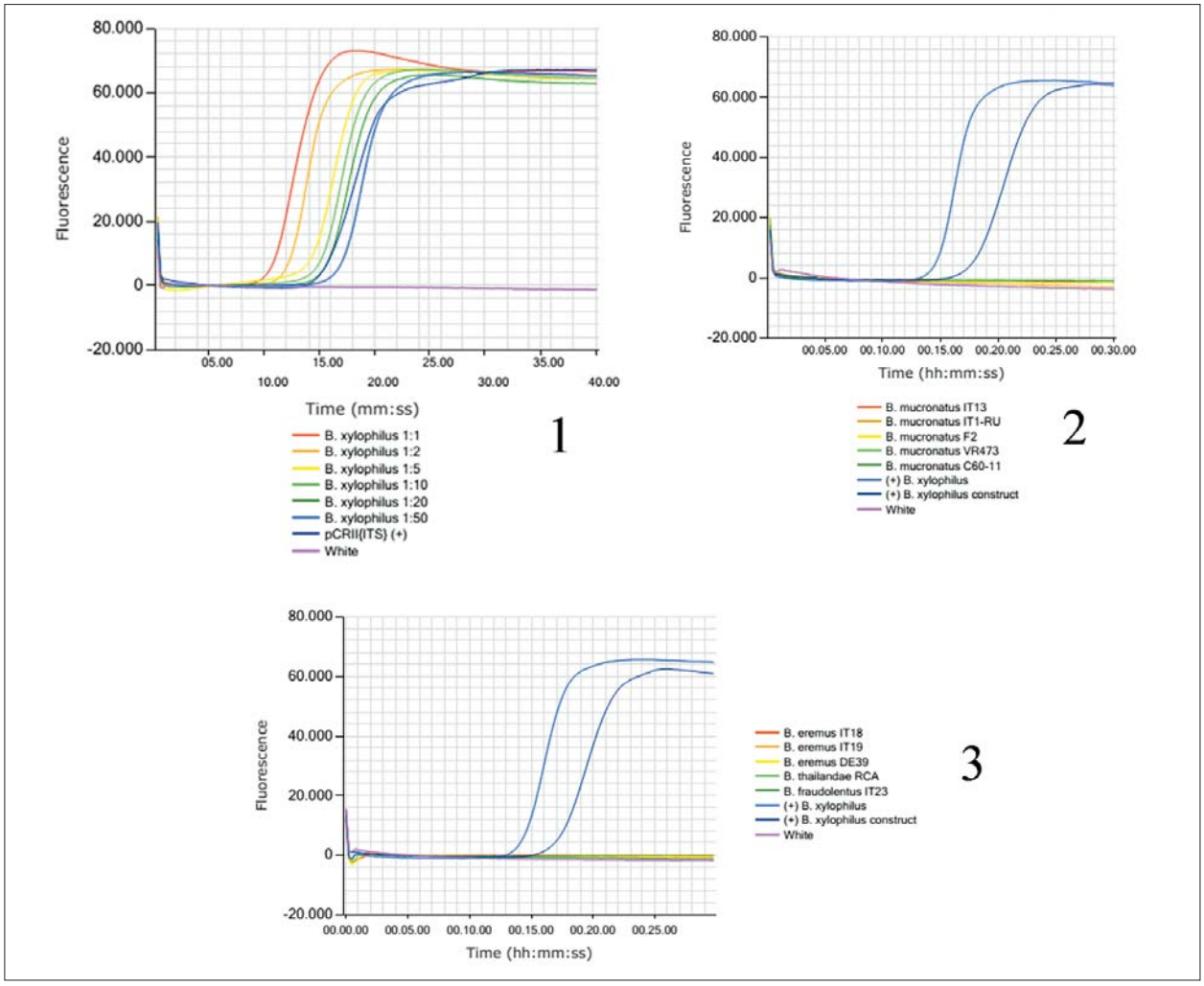

Fig. III – Sensibility and specificity of diagnostic test in the conditions used. 1. Detection of single nematode *Bursaphelenchus xylophilus* DNA at diluitions tested. 2. Discrimination between *B. xylophilus* DNA from *B. mucronatus*. 3. Specific identification of *B. xylophilus* DNA from other *Bursaphelenchus* species.