



HAL
open science

Early detection of *Aspergillus carbonarius* and *A. niger* on table grape: a tool for quality improvement

Fatima Ayoub, Massimo Reverberi, Alessandra Ricelli, Anna Maria d'Onghia, Thaer Yaseen

► To cite this version:

Fatima Ayoub, Massimo Reverberi, Alessandra Ricelli, Anna Maria d'Onghia, Thaer Yaseen. Early detection of *Aspergillus carbonarius* and *A. niger* on table grape: a tool for quality improvement. *Food Additives and Contaminants*, 2010, 27 (09), pp.1285-1293. 10.1080/19440049.2010.489576 . hal-00603431

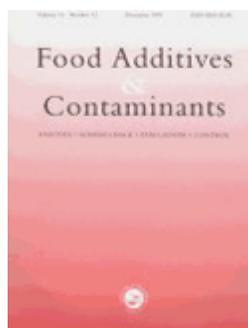
HAL Id: hal-00603431

<https://hal.science/hal-00603431>

Submitted on 25 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Early detection of *Aspergillus carbonarius* and *A. niger* on table grape: a tool for quality improvement

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2009-394.R1
Manuscript Type:	Special Issue
Date Submitted by the Author:	24-Mar-2010
Complete List of Authors:	Ayoub, Fatima; Centre International de Hautes Etudes Agronomiques Méditerranéennes (CIHEAM/MAIB), IPM reverberi, massimo; Università La Sapienza, Plant Biology Ricelli, Alessandra; Istituto di Chimica Biomolecolare-CNR D'Onghia, Anna; Centre International de Hautes Etudes Agronomiques Méditerranéennes (CIHEAM/MAIB), IPM Yaseen, Thaer; Centre International de Hautes Etudes Agronomiques Méditerranéennes (CIHEAM/MAIB), IPM
Methods/Techniques:	Molecular biology - PCR, HPLC
Additives/Contaminants:	Mycotoxins - fungi, Ochratoxin A
Food Types:	Fruit

SCHOLARONE™
Manuscripts

Food Additive and Contaminants,

Early detection of *Aspergillus carbonarius* and *A. niger* on table grapes: a tool for quality improvement

Fatima Ayoub^a, Massimo Reverberi^b, Alessandra Ricelli^c, Anna Maria D'Onghia^a and Thaer Yaseen^{a*}

^a*Centre International de Hautes Etudes Agronomiques Méditerranéennes (CIHEAM/MAIB), Via Ceglie 9, 70010 Valenzano, Bari, Italy.*

^b*Dipartimento Biologia Vegetale Sapienza Università di Roma L.go C. di Svezia 24, 00165, Roma, Italy.*

^c*Istituto di Chimica Biomolecolare-CNR Piazzale A. Moro 5, 00185 Roma, Italy.*

Aspergillus carbonarius and *A. niger* aggregate are the main fungal contaminants of table grapes. Besides their ability to cause the Black rot, they can produce Ochratoxin A (OTA), a mycotoxin that has attracted increasing attention worldwide. The objective of this work was to set up a simple and rapid molecular method for the early detection of both fungi in table grapes before fungal development becomes evident. PCR-based assays were developed by designing species-specific primers based on the polyketide synthases (PKS_S) sequences of *A. carbonarius* and *A. niger* that have recently been demonstrated to be involved in OTA biosynthesis. Three table grape varieties (Red globe, Crimson seedless and Italia) were inoculated with *A. carbonarius* and *A. niger* aggregate strains producing OTA. The extracted DNA from control (non-inoculated) and inoculated grapes was amplified by PCR using ACPKS2F-ACPKS2R for *A. carbonarius* and ANPKS5-ANPKS6 for *A. niger* aggregate. Both primers allowed a clear detection even in symptomless samples. Since the high specificity and sensitivity of PCR-based methods, they are considered to be a good alternative to traditional diagnostic means for the early detection of fungi in complex matrix. The results obtained could be useful for the definition of a “quality label” for tested grapes to improve the safety measures taken to guarantee the production of fresh table grapes.

Key words: *Aspergillus carbonarius*, *Aspergillus niger*, Ochratoxin A, Polyketide synthase, Real time PCR, Table grapes

Introduction

Many fungi may occur on table grapes in the vineyard, but the main concern from the point of view of the mycotoxin contamination is represented by the group of black *Aspergillus* species, in particular *Aspergillus carbonarius* and *Aspergillus niger* aggregate (Ailsa et al. 2007). They are *Deuteromycotina* fungi (Imperfect fungi, asexual reproduction) that belong to the order of *Hyphomycetes*, family of *Moniliaceae*, section *Nigri* (Atoui, 2006). In addition to their ability to cause Black rot diseases in the field, they can produce ochratoxin A (OTA), a mycotoxin that is attracting increasing attention worldwide (Varga et al, 2006).

Ochratoxin A, the most toxic of the ochratoxins, is produced by several fungal species belonging to *Aspergillus* and *Penicillium* genera (Varga et al. 2006). This mycotoxin has a significant economic impact and poses a serious problem for human health. It is named after *Aspergillus ochraceus*, the first rot agent from which it was isolated (Van der Merwe et al, 1965), although later on other genera were reported to be capable of producing this toxin. The compound 7-carboxyl-5-chloro-3,4-dihydro-8-hydroxyl-3*R*-methylisocoumarin-7-*L*-phenylalanine (C₂₀H₁₈ClNO₆), usually known as OTA, can be found in several food and drink commodities, including cereals, coffee and wine, at different levels (Battaglia et al. 1996).

Several studies have been performed to investigate the contamination with black aspergilli on wine grapes (Battilani et al. 2004, Leong et al. 2006), but only few works were carried out on table grapes (Guzev et al. 2006). In this work *A. carbonarius* and *A. niger* aggregate strains able to produce OTA were frequently isolated from table grapes.

Most research efforts were concentrated on the means for prevention of OTA biosynthesis as the best defence for protecting the consumer. However, prevention is not always possible, especially from OTA biosynthesis occurring under field conditions. In fact, OTA remains in processed food and the recovery of infected products is only possible through their decontamination (Bellì, 2006). Conversely, in the case of table grapes, the prevention remains the only possible way to control OTA formation; this point can be achieved only thanks to the early detection of the OTA-producer fungus.

1
2
3 Traditional diagnostic methods used in food mycology are based on macroscopic and
4 microscopic features and culture in appropriate media. On the one hand, the development of
5 fruiting structures requires 2 to 10 days of culture on different media (Raper and Fennell,
6 1965), increasing considerably the time of analysis. On the other hand, these methods have
7 shown a low degree of sensitivity and are difficult to standardize (Zhao et al, 2001).
8 Misidentification can often occur because some fungi may be poorly characterized or because
9 a considerable expertise is required (Patiño et al, 2005).
10
11
12
13
14
15
16
17

18 The increasing use of molecular methods in fungal diagnosis has provided tools for answering
19 taxonomic questions that morphological procedure have left unsolved (Taylor et al, 1999,
20 Parenicova et al. 2000). PCR-based methods that target on DNA are considered a good
21 alternative for rapid and early diagnosis because of their high specificity and sensitivity
22 (Bluhm et al, 2002).
23
24
25
26
27
28
29

30 Reduction of OTA contamination may be accomplished by the early detection of OTA-
31 producing fungal species in order to intervene at the right time to reduce the source of OTA.
32
33

34 Several approaches have been followed for the molecular detection of the OTA-producing
35 fungi. Atoui et al. (2006) developed a specific PCR assay for the detection of *A. carbonarius*
36 in grapes, grape juices and wines.
37
38
39
40
41

42 González-Salgado et al. (2009) set up a specific detection method of some *A. carbonarius*
43 strains isolated from Portugal by SYBR Green and TaqMan quantitative PCR assays based on
44 the multicopy ITS2 region of the rRNA gene to be used in wine grapes. The primers and the
45 TaqMan probe were based on the ITS 2 multicopy region. The specificity and sensitivity of
46 both assays were tested on genomic DNA mixtures of several *A. carbonarius* strains and other
47 fungal species frequently present in grapes. Both methods were also compared using grapes
48 inoculated with different spore concentrations of *A. carbonarius*, detecting up to 0.4 pg
49 DNA/g of berries.
50
51
52
53
54
55
56
57
58
59
60

1
2
3 As for *A. niger*, no data have been found so far in the literature for detection and
4
5 quantification using real-time PCR assays on grapes.
6

7 For this reason, the objective of this work is to set-up a simple, rapid, specific and sensitive
8
9 molecular method for the early detection of *A. carbonarius* and *A. niger* aggregate in table
10
11 grapes, before fungal development becomes evident.
12

13 14 15 **Materials and methods**

16 17 *Fungal strains and culture conditions*

18
19 Each grape sample (~500 g) was washed with 500 ml of sterile distilled water on a shaker for
20
21 30 min. Aliquots (100µl) of washing water and its serial dilution were spread on Czapek-Dox-
22
23 Agar medium (CDA) with 0.02% of Streptomycin sulphate.. The plates were incubated at 25°C
24
25 and daily checked until fungal colonies formation. Each colony was transferred on a Petri dish
26
27 with the same medium and incubated for 7 days at 25°C. *Aspergillus* strains were identified
28
29 according to their morphological characteristics (macroscopically and microscopically),
30
31 following the rules and descriptions provided by Raper and Fennell (1965) and Pitt and
32
33 Hocking 1997 and in the other hand following molecular tools. The strains were also tested to
34
35 determine their ability to produce OTA. The black aspergilli strains have been preserved in
36
37 slants with CDA medium at 4°C.

38
39 All strains used in this study were OTA producers. The different strains were isolated from
40
41 grapes in Apulia, Italy. Fungal cultures were maintained on CDA medium and stored on the
42
43 same medium at 4°C.

44 45 *Grapes infection*

46
47 Berries from three table grape varieties, Red globe, Crimson seedless and Italia, were
48
49 collected during harvesting time and stored at 4°C. Grapes were superficially sterilized with
50
51 sodium hypochlorite at 2% v/v for 2 minutes, rinsed with sterile distilled H₂O and incubated
52
53 in container at 30°C after superficial inoculation by 20µl of conidia suspension (2.5 CFU/µl)
54
55 prepared from OTA producer strain of *A. carbonarius* or *A. niger* aggregate. Four different
56
57 experiments have been performed during 40 days of post-harvest storage: at harvest, after 13,
58
59 26 and 40 days of conservation. . Inoculated and un-inoculated berries samples were collected
60
61 0, 3, 6, 9 and 12 days after inoculation and stored at - 80°C until the day of analysis.

DNA extraction from fungal strains

Aspergillus carbonarius and *A. niger* aggregate strains were cultured in 100 ml Erlenmeyer flasks containing 20 ml Potato Dextrose-Broth with 4-5 mycelial disks cut from the border of 4 to 5-days old colonies on PDA plates, and incubated at 26°C on shaker at 120 rpm. Mycelia from 4 days-old cultures were harvested by filtration through Whatman paper n° 1 and kept at -20°C for DNA extraction. Five to ten mg of the conserved mycelia were collected with a spatula, suspended in 400 µl of breaking buffer and extracted with 400 µl of Phenol/Chlorophorm/Isoamyl alcohol (25:24:1 v/v/v) in the presence of 25 mg of acid washed glass beads (425-600 µm diameter) and two sterile 5 mm stainless steel balls. This mixture was vortexed at 3,000 rpm for 10 min, and centrifuged for 15 min at 13,000 rpm. The supernatant was mixed with an equal volume of Chloroform/Isoamyl alcohol (24:1 v/v), vortexed at 3,000 rpm for 2 min, centrifuged for 5 min at 13,000 rpm and precipitated with two volumes of 100% cold isopropanol (-20°C). The precipitated DNA was washed with 70% (-20°C) ethanol, dissolved in 50 µl of TE buffer (10 mM Tris-HCL, 0.1 Mm EDTA, pH 7.6) and quantified using a spectrophotometer (Yaseen et al. 2004).

DNA extraction from grape samples

An aliquot of 250 mg of lyophilized homogenized grapes were suspended in 1 ml of lysis buffer (200 mM of Tris HCl pH 8, 25 mM of NaCl, 25 mM of EDTA and 0.5% of SDS) and vortexed at 3,000 rpm for 10 min. After 1 hour at 65°C, and 10 minutes in ice, the sample was centrifuged at 13,000 rpm at 4°C for 15 minutes. The aqueous phase was mixed with 200 µl of sodium acetate 4M, put in ice for 30 minutes and centrifuged at 13,000 rpm at 4°C for 15 minutes. The recovered supernatant was homogenized with an equal volume of Phenol/Chloroform/Isoamyl alcohol (25:24:1 v/v/v), vortexed for 5 minutes and centrifuged at 13,000 rpm at 4°C for 10 min. An equal volume of chloroform 100% was added to the aqueous phase, then gently vortexed for 5 min and centrifuged at 12,000 rpm at 4°C for 15 min.

The DNA contained in the aqueous phase was precipitated by adding half a volume of Isopropanol and stored for 20 min at -20°C. The pellet obtained after 15 minutes of centrifuge at 13,000 rpm at 4 °C was washed with ethanol 70% and centrifuged at 13,000 rpm for 15 min at 4°C. The pellet obtained was dried, dissolved in 28 µl of sterile distilled H₂O and 2 µl of RNase (20 mg/ml) and stored at 37°C for 30 min.

Primer design and PCR reaction

Aspergillus carbonarius-specific primer

PKS genes encode the polyketide synthase (PKS) enzyme, which is involved in the biosynthesis of secondary metabolites. In the gene bank, there are only five partial sequences of *A. carbonarius* PKS gene (Atoui et al. 2006). On the basis of these sequences, a degenerated primer (PKS1) for *Aspergillus* spp. and a specific primer for *Aspergillus carbonarius* (ACPKS2) were designed (Table 1).

Aspergillus niger-specific primer

The degenerated primer for *Aspergillus* spp. PKS1 was used to amplify a partial sequence of the PKS gene of *A. niger* (Yaseen et al, 2009). On the basis of this sequence, a couple of primers have been designed to amplify a DNA fragment of 157 bp: ANPKS 5 (5' ACGGTAAACGTCCTGGATGA 3') and ANPKS 6 (3' CGTGCTGTTGAAGCCACTT 5').

PCR amplification

Specific PCR assays have been developed in this study for the detection of both *A. carbonarius* and *A. niger*, the main source of OTA contamination on table grapes. To demonstrate the specificity of these primers, PCR amplification of genomic DNA from various fungal strains has been performed; PCR was executed in 25 µl mixture containing 100ng of genomic DNA, 5 µl of 5x Colorless Go Taq Flexi buffer (Promega, Madison WI-U.S.A), 100 µM of dNTPs buffer (Promega, Madison WI-U.S.A), 2.5 mM MgCl₂ (Promega, Madison WI-U.S.A), 10 µM each of forward and reverse primers and 2.5 unit of GoTaq® DNA polymerase (5 U/µl Promega, Madison WI-U.S.A). The reactions were performed under the following PCR conditions: 95°C for 300 s; 35 cycles at 95°C for 10 s, 62°C for 10 s for *A. carbonarius* and 52°C for *A. niger*, 72°C for 15 s; plus a final extension cycle at 72°C for 300 s. To visualize the amplification of the expected size fragment, 8 µl of each PCR sample was analyzed by agarose gel electrophoresis. Amplicons were separated by electrophoresis in 1.5% agarose gels in TAE buffer (1x) at 80V for 10 600 s followed by 100 V for 45 min, and stained for 10 min with ethidium bromide (2 µg ml⁻¹).

Quantitative detection by Real-time PCR

A Real-time PCR method was developed using SYBR Green dye to detect *A. carbonarius* and *A. niger* OTA-producing strains on table grapes. DNA extractions were performed from 191 samples (from the three tested varieties), and subject to real-time PCR amplification. Quantitative real-time PCR reaction was incubated in a programmable thermal cycler (iQ5-Bio-rad) starting from 5 min denaturation at 95°C followed by 35 cycles at 95°C for 10 s, annealing at 62°C for 10 s for *A. carbonarius* and 52°C for *A. niger*, extension at 72°C for 15 s. Reactions were performed in 25 µl mixture containing: 12.5 µl of 1X iQSYBR Green Supermix (Bio-Rad), 10µM forward and reverse primers, 5 ng of DNA template and deionised sterilized water up to 25 µl. Standard curve was generated from the amplification of ten-fold dilutions of *A. carbonarius* and *A. niger* DNA ranging from 10 ng to 0.01ng. These DNA amounts were subject to real-time PCR conjugated with SYBR Green dye using the specific primers ACPKS2 F/R for *A. carbonarius* and ANPKS 5/6 for *A. niger*. The parameter used for data interpretation was the threshold cycle (Ct value), which represents the cycle number at which the fluorescence emission exceeds the fixed threshold. Quantification of the unknown DNA samples was carried out by interpolating the samples Ct values with the standard curves (Atoui et al, 2006). At the end of each run, and to ensure that only the specific PCR product was present, a Melting curve was performed which was programmed as 81 cycles of 30 seconds at 55 °C.

Results and discussion

Specificity of the PCR reaction

A single fragment of 330 bp of the target DNA was obtained only when genomic DNA from *A. carbonarius* was used with ACPKS2 (F/R) and of 157 bp from *A. niger* with ANPKS (5/6) primers (Figure 1 A/B). The absence of any aspecific band using DNA extracted from other *Aspergillus* spp. and from filaments fungi demonstrate the specificity of primers, and the possibility of application of quantities real time PCR.

1
2
3 ***Development of the Real-time PCR method for *A. carbonarius* and *A. niger* DNA***
4 ***quantification***
5
6

7 A standard curve was obtained by plotting the Ct value versus the logarithm of the
8 concentration of each DNA dilution. The linear correlation coefficient of the standard curve
9 was $R^2 = 0.963$ and $R^2 = 0.993$ for *A. carbonarius* (Figure 2/A) and *A. niger* (Figure 2/B)
10 respectively, demonstrating the accuracy of PCR-based quantification. Consequently, the use
11 of the generated standard curves allows quantifying DNA of *A. carbonarius* and *A. niger* from
12 contaminated table grape vine.
13
14
15
16

17
18 ***Application of the qPCR method for *A. carbonarius* and *A. niger* in artificially***
19 ***contaminated samples*** by interpolating its Ct value against the standard curve. The results
20 were expressed in ng DNA/ μ l. The amount of DNA of both fungi did not statistically change
21 until 26 days of post-harvest storage at 4°C, then the quantity of DNA of both fungi
22 significantly increased after 40 days of storage in case of Italia and Red Globe but not in
23 Crimson (Figure 3). These results could be partly explained considering the post-harvest
24 aging processes. In fact, during storage time, the grape berries become more susceptible to the
25 attack by these fungi. In addition, these results are correlated with those of visual infection,
26 showing a high percentage of infection: up to 70% in berries stored for 40 days then incubated
27 at 30°C for 12 days. Hence, the development of this fungus was much rapid reaching high
28 percentage of infection after 26 days of post-harvest cold storage. A gradual increase in the
29 DNA content of *A. carbonarius* and *A. niger* in each variety during the incubation periods at
30 30°C (0, 3, 6, 9 and 12 days) was monitored after 40 days of post-harvest cold storage (Figure
31 4 A/B). The target fungal DNA, of both *A. carbonarius* and *A. niger* was detected even at time
32 0 (without incubation), the lowest detection limit achieved was $2.37 \cdot 10^{-2}$ ng/ μ l showing the
33 high sensitivity of the detection method (Figure 4 A/B).
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

50 Optimization of a Real-time PCR assay is very important, mainly when SYBR Green dye is
51 used. The analysis of the melting curve and the parameters calculated from the standard curve
52 showed that the designed method was highly optimized. The melting curve for *A. carbonarius*
53 showed that only one fragment was amplified and primers did not form other aspecific bands
54 (data not shown).
55
56
57
58
59
60

1
2
3 It was evident that both *A. carbonarius* and *A. niger* were detected in all samples, nevertheless
4 a superficial sterilization was performed. These results correlate with the visual observations
5 (Figure 5); fungal growth was observed not only nearby the zone of inoculation, but also
6 around the stalk of each berry, where, usually, the fungus starts to develop in non inoculated
7 samples. Moreover, according to Battilani et al. 2003, *A. carbonarius*, as well as *A. niger* can
8 enter the berries even without skin damage.
9
10
11
12
13
14
15
16

17 Several amplification methods have been developed with conventional PCR for the analysis
18 of phytopathogenic fungi (competitive PCR) (Hadidi et al. 1995; Mahuku and Platt, 2002).
19 However, these methods are laborious and insufficiently accurate because the amplification
20 efficiency decreases in later PCR cycles (Ginzinger, 2002). An accurate, reliable, and high-
21 throughput quantification of target DNA requires a real-time PCR approach (Lie and
22 Petropoulos, 1998; Schmittgen, 2001). Real-time PCR quantifies initial concentrations of
23 DNA by the threshold cycle (Ct), the amplification cycle at which the concentration of DNA
24 produced in the PCR reaction gives a visualized fluorescence climbs above a baseline
25 “threshold”. Therefore, a lower Ct corresponds to higher amount of DNA in the beginning of
26 the reaction.
27
28
29
30
31
32
33
34
35
36

37 Concentration values obtained by Real-time PCR in samples with a starting quantity of 5 ng
38 of total DNA extracted from samples were related with the initial amounts of *A. carbonarius*
39 and *A. niger* DNA. Moreover, the application of the method to quantify these fungi in
40 artificially contaminated matrices was confirmed and detection was executed at longer
41 incubation times at 30 °C.
42
43
44
45
46
47
48

49 The specificity and high degree of sensitivity of real time PCR detection method developed
50 for *A. carbonarius* and *A. niger* provide a good tool for the early detection of this OTA
51 producing fungi in table grapes and to prevent OTA entering the food chain. Detection of
52 these fungi, in the case of table grapes, becomes particularly critical around harvest time when
53 the OTA production is considered high.
54
55
56
57
58
59
60

1
2
3 In this work we described a real-time PCR method that lead to the identification and
4 quantification of *Aspergillus carbonarius* and *Aspergillus niger* aggregate in three varieties of
5 table grapes without visible symptoms of rotting. This method was rapid, specific with high
6 sensitivity, providing a useful tool for early detection of both fungus and prediction of OTA
7 contamination in table grapes susceptible to be colonized by these species. Moreover, the
8 results obtained could be useful to define a “quality label” for the assayed grapes in order to
9 improve the safety measures taken to guarantee the production of fresh table grapes.
10
11
12
13
14
15
16
17

18 **References**

19
20 Ailsa DH, Su-lin LL, Benozir AK, Robert WE Eileen SS. 2007. Fungi and mycotoxins in
21 vineyards and grape products. *Int J Food Microbiol.* 119: 84–88.
22
23

24 Atoui A. 2006 Approche de la mycotoxinogenese chez *Aspergillus ochraceus* et *Aspergillus*
25 *carbonarius*: Etudes moleculaire et physiologique. Ph.D thesis. ENSAT, France.
26
27

28 Atoui A, Mathieu F, Lebrihi A. 2006. Targeting a polyketide synthase gene for *Aspergillus*
29 *carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. *Int J*
30 *Food Microbiol.* 115: 313-318.
31
32
33

34 Battaglia R, Hatzold T, Kroes R. 1996. Occurrence and significance of ochratoxin A in food.
35 *Food Add Contam,* 13: 1-3.
36
37

38 Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z. 2003. Occurrence of
39 ochratoxin A-producing fungi in grapes grown in Italy. *J Food Protect* 66: 633–636.
40
41

42 Battilani P, Logrieco A, Giorni P, Cozzi G, Bertuzzi T, Pietri A. 2004. Ochratoxin A
43 production by *Aspergillus carbonarius* on some grape varieties grown in Italy. *Journal of the*
44 *Science of Food and Agriculture.* 84(13): 1736-1740.
45
46
47

48 Bellí N. 2006. Ochratoxin A and ochratoxigenic moulds in grapes, must and wine.
49 Ecophysiological studies. Ph.D thesis. Universitat de Lleida, Lleida, Spain..
50
51

52 Bluhm BH, Flaherty JE, Cousin MA Woloshuk CP. 2002. Multiplex polymerase chain
53 reaction assay for the differential detection of trichothecene- and fumonisin-producing
54 speciesof *Fusarium* in cornmeal. *J Food Protect.* 65: 1955–1961.
55
56
57

58 Ginzinger DG. 2002. Gene quantification using real-time quantitative PCR: an emerging
59 technology hits the main stream. *Experiment Hematol.*30: 503-512.
60

1
2
3 González-Salgado A, Patiño B, Gil-Serna J, Vázquez C, González-Jaén MT. 2009. Specific
4 detection of *Aspergillus carbonarius* by SYBR Green and TaqMans quantitative PCR assays
5 based on the multicopy ITS2 region of the rRNA gene. FEMS Microbiol Lett. 295: 57-66
6
7

8
9 Guzev L, Danshin A, Ziv S, Lichter A. 2006. Occurrence of ochratoxin A producing fungi in
10 wine and table grapes in Israel. Int J Food Microbiol. 111: S67-71.
11

12
13 Hadidi A, Levy L, Podleckis EV. 1995. Molecular Methods in Plant Pathology. CRC Press
14 Inc., London, UK: R.P Singh, U.S. Singh. Polymerase chain reaction technology in plant
15 pathology. p. 167-187.
16
17

18
19 Leong SL, Hocking AD, Scott ES. 2006. Survival and growth of *Aspergillus carbonarius* on
20 wine grapes before harvest. Int J Food Microbiol. 111(1): S83-S87.
21

22
23 Lie YS, Petropoulos CJ. 1998. Advances in quantitative PCR technology: 5' nuclease assays.
24 Curr Op in Biotechnol. 9: 43-48.
25

26
27 Mahuku GS, Platt H.W. 2002. Quantifying *Verticillium dahliae* in soils collected from potato
28 fields using a competitive PCR assay. Am J Potato Res. 97: 107-117.
29

30
31 Parenicova L, Skouboe P, Samson RA, Rossen L, Visser J. 2000. Integration of modern
32 taxonomic methods of *Penicillium* and *Aspergillus* classification. Hardwood Academic
33 Publishers, Amsterdam, the Netherlands: Samson RA and Pitt JI. Molecular tools for the
34 classification of black Aspergilli. p. 413-424.
35
36
37

38
39 Patiño B, González-Salgado A, González-Jaén MT, Vázquez C. 2005. PCR detection assays
40 for the ochratoxin-producing *Aspergillus carbonarius* and *Aspergillus ochraceus* species. Int
41 JFood Microbiol. 104: 207-214.
42

43
44 Pitt JI, Hocking AD. 1996. Current knowledge of fungi and mycotoxins associated with food
45 commodities in southeast Asia. Paper presented at 17th Asean Technical Seminar on Grain
46 Postharvest Technology- Mycotoxin contamination in grains Lumut, Malaysia Book Series:
47 Aciar Technical Reports (37) p. 5-10.
48
49

50
51 Raper KB, Fennell DI. 1965. The genus *Aspergillus*. New York, USA: Williams and Wilkins.,
52

53
54 Schmittgen TD. 2001. Real-time quantitative PCR. Methods 25: 383-385.
55

56
57 Taylor JW, Geiser DM, Burt A, Koufopanou V. 1999. The evolutionary biology and
58 population genetics underlying fungal strain typing. Clinical Microbiol Rev. 12: 126-146.
59
60

1
2
3 Van der Merwe KJ, Steyn PS, Fourie L. 1965. Ochratoxin A, a toxic metabolite produced by
4 *Aspergillus ochraceus*. Wilh., Nature 265:1112-1113.
5
6

7 Varga J, Kozakiewicz Z. 2006. Ochratoxin A in grapes and grape derived products. Trends in
8 Food Sci & Technol. 17: 72–81.
9
10

11 Yaseen T, Ippolito A, Faretra F, Casulli F. 2004. Molecular diagnosis and biological control
12 of Phytophthora-citrus root rot. Ph.D thesis. Bari: University of Bari.
13
14

15 Yaseen T, Ayoub F, Ricelli A. 2009. Partial polyketide synthases sequence of *A. niger* and
16 specific PCR based detection. Paper presented at ISM Conference 2009. Proceedings of the
17 ISM Conference Tulln/Vienna, Austria.
18
19
20

21 Zhao J, Kong F, Li R, Wang X, Wan Z, Wang D. 2001. Identification of *Aspergillus*
22 *fumigatus* and related species by nested PCR targeting ribosomal DNA internal transcribed
23 spacer regions. J Clinical Microbiol. 39: 2261-2266.
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 **Figure 1.** Agarose gel electrophoresis of amplification products from genomic DNA of
4 various fungal isolates obtained with primer pairs ACPKS2 (F/R) specific for *A. carbonarius*,
5 amplifying a fragment of 330 bp (A); primer pairs ANPKS 5/6 specific for *A. niger*,
6 amplifying a fragment of 157bp. Lanes from 1 to 17 are *Alternaria* sp., *Botrytis cinerea*,
7 *Cladosporium* sp., *Cryphonectria parasitica*, *Cylindrocarpon* sp., *Gliocladium* sp.,
8 *Cladosporium* sp., *Colletotrichum* sp., *Penicillium* sp., *Phomopsis viticola*, *Sclerotinia*
9 *sclerotiorum*, *Dothiorella ribis*, *Septoria tritici*, *Trichoderma* sp., *Phoma* sp., *Macrophomina*
10 sp. and *A. niger* respectively. Lane 18/A: *A. carbonarius* and lane 19/A: Non-template
11 control, lane 18/B: Non-Template control and lane 19/B: *A. niger*.
12
13
14
15
16

17 **Figure 2.** Correlation between Log starting quantity of DNA ng/μl and cycle threshold (Ct)
18 value as assessed by SYBR green Real-time PCR of *A. carbonarius* (A) and of *A. niger* (C).
19 Standard curve generated from the amplification of ten-fold dilutions (from 10 ng/μl to 0.01
20 ng/μl) from target genomic DNA of *A. carbonarius* (B) and of *A. niger* (D), the coefficient of
21 linear regression equation of Ct with Log DNA concentrations was $R^2 = 0.963$ and $R^2 = 0.993$
22 respectively.
23
24
25

26 **Figure 3.** Detection of DNA quantity of *Aspergillus carbonarius* and *A. niger* by SYBR green
27 Real-time PCR extracted from inoculated and non-inoculated table grapes of Red globe, Italia
28 and Crimson seedless, starting from the day of harvest and during 40 days of post-harvest
29 cold storage. Columns report average of DNA content from four different DNA extractions
30 and amplifications. Bars represent standard deviation.
31
32

33 **Figure 4.** DNA quantification of *A. carbonarius* (A) and *A. niger* (B) by SYBR green Real-
34 time PCR using ACPKS2 (F/R) and ANPKS (5/6) specific primers, respectively, of inoculated
35 and non-inoculated (control) table grapes of Red globe, Italia and Crimson seedless, during
36 the incubation periods at 30°C (0, 3, 6, 9 and 12 days) after 40 days of post-harvest cold
37 storage.
38

39 **Figure 5.** Percentage of infected grapes Red globe (R), Italia (I) and Crimson seedless (C)
40 cultivars with *A. carbonarius* (c) and *A. niger* (n), after 0 (A), 13 (B), 26 (C) and 40 (D) days
41 of postharvest cold storage during different incubation periods (0, 3, 6, 9 and 12 days) at 30
42 °C. Un-inoculated grapes from each cultivar were used as controls (t).
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Sequence of degenerated primers for *Aspergillus* spp. (PKS1) and specific primers for *A. carbonarius* (ACPKS2).

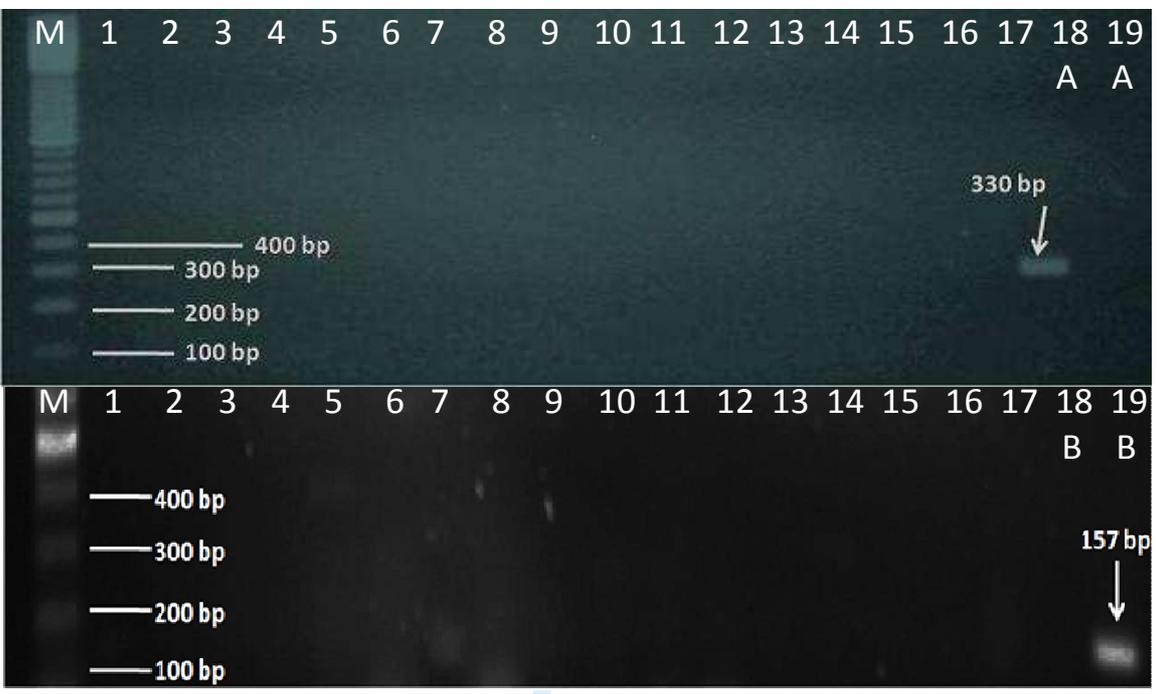
Primer	Forward 5'-3'	Reverse 3'-5'	Fragment
PKS1	GATGAGAA(CT)GC(TA)GATGG(GC)T	TCGCCGA(GA)(TC)TGGGTGCCGGTGCC	267 bp
ACPKS2	GCAGCGGGAGTCAATGTAAT	GCGTCGTACAAAGCCTCTT	330 bp

Deleted: b

Deleted: b

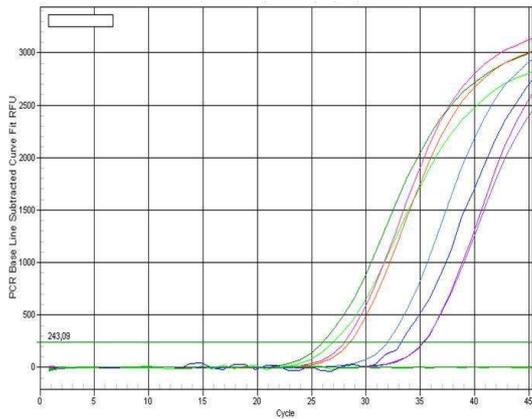
For Peer Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

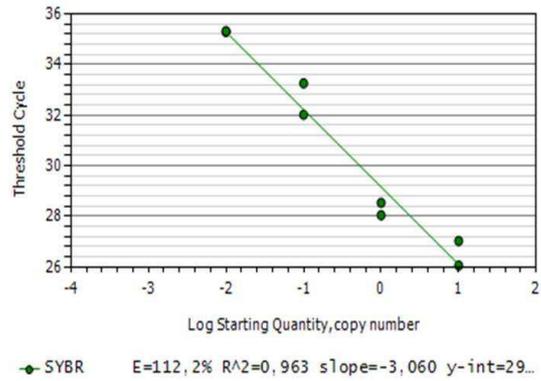


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

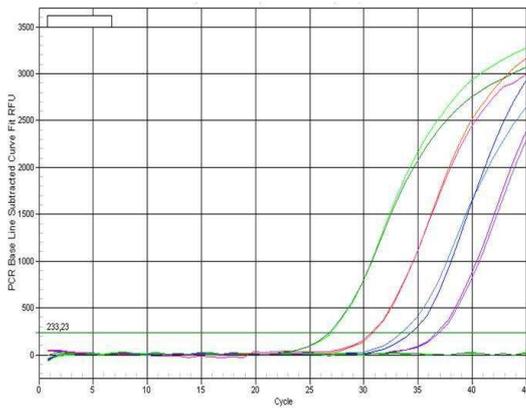
A



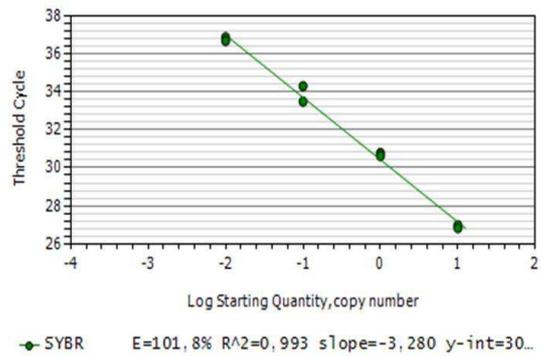
B



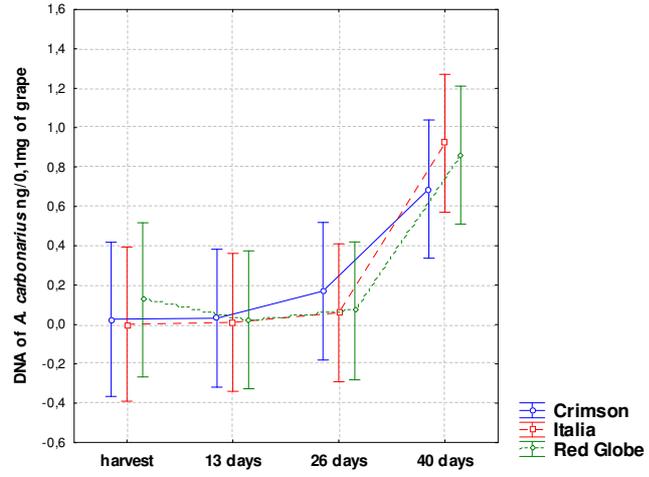
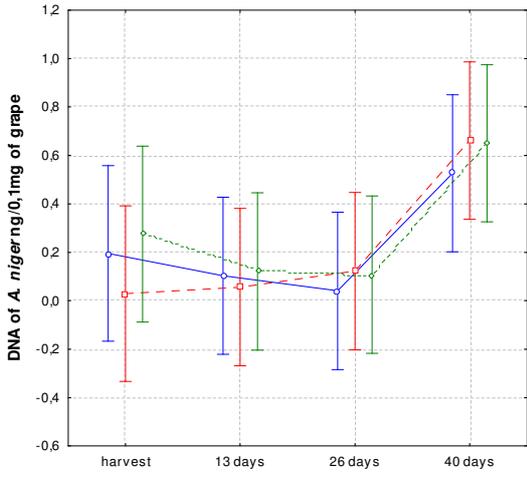
C



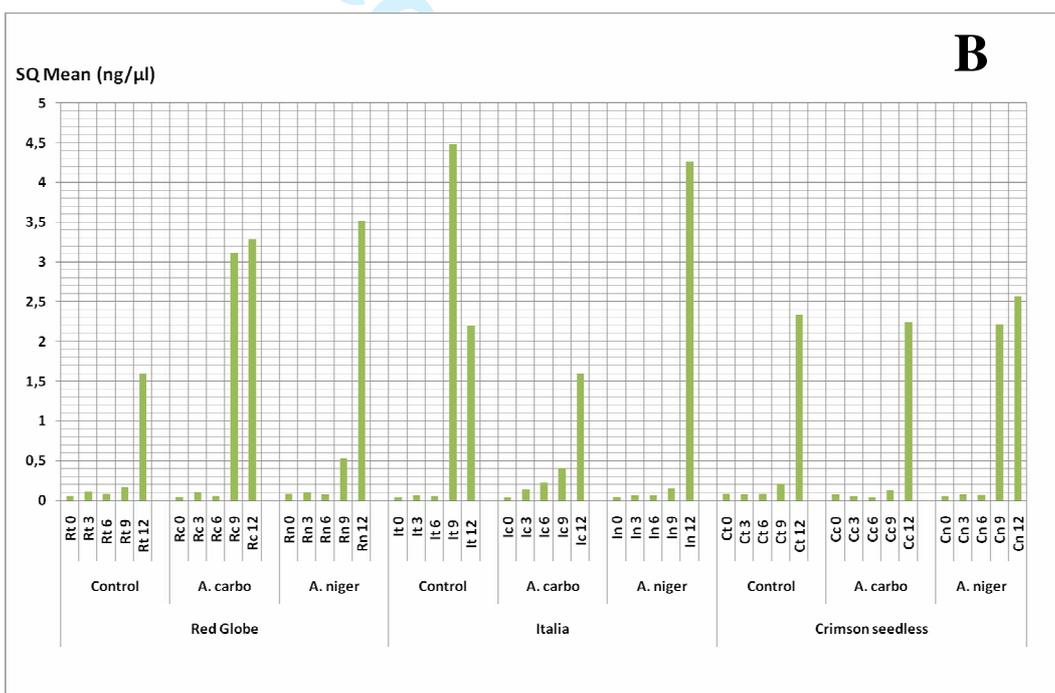
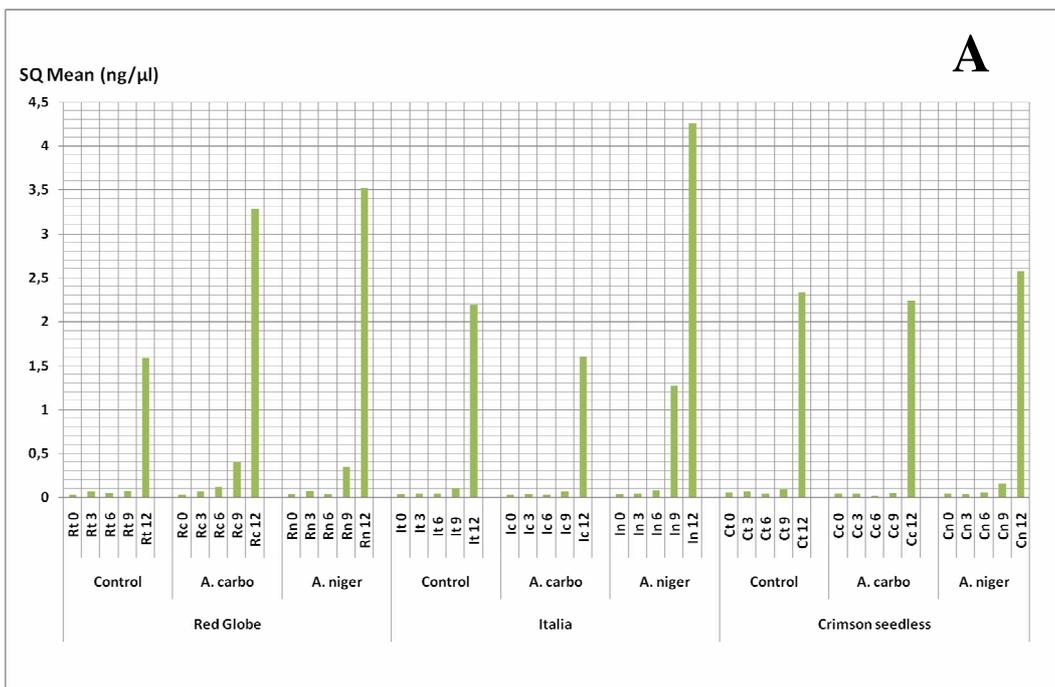
D

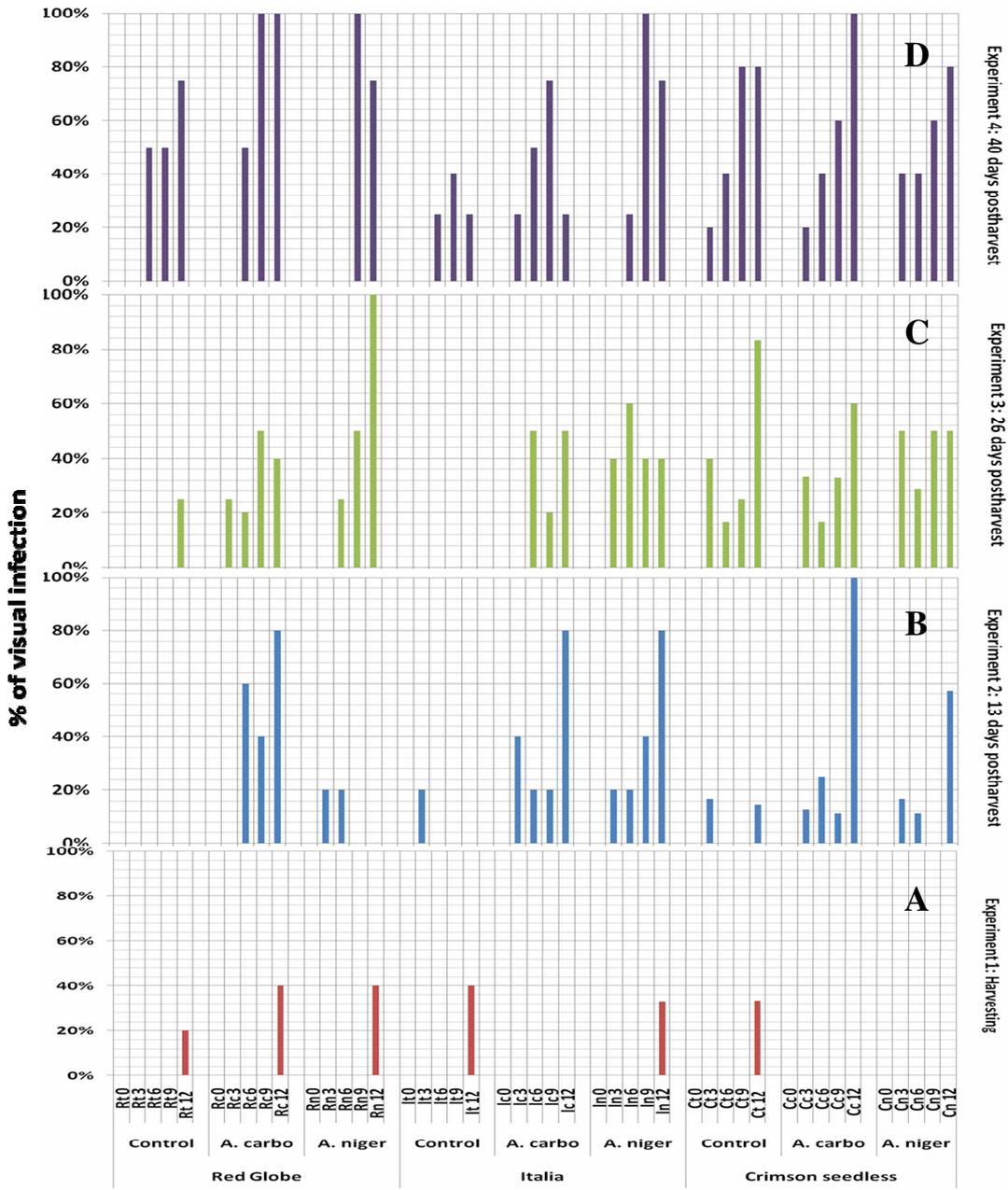


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



Peer Review Only





1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60