JOURNAL OF PLANT PROTECTION RESEARCH Vol. 56, No. 3 (2016)
DOI: 10.1515/jppr-2016-0036

Discoloured seeds of amaranth plant infected by *Alternaria alternata*: physiological, histopathological alterations and fungal secondary metabolites associated or registered

María Cristina Noelting[1], Marina Sisterna[2,3], Marcelo Lovisolo[4], Andrés Molla-Kralj[4], Gladys Lori[2,3], María Cristina Sandoval[4], Michael Sulyok[5], Maria C. Molina[1,6]*

[1] Instituto Fitotécnico de Santa Catalina, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CP 1836, Llavallol, Buenos Aires, Argentina
[2] Centro de Investigaciones Fitopatológicas, Cátedra de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CP1900 La Plata, Buenos Aires, Argentina
[3] Comisión de Investigaciones Científicas de la provincia de Buenos Aires, CP 1900 La Plata, Buenos Aires Argentina
[4] Facultad de Ciencias Agrarias, Universidad Nacional de Lomas de Zamora, CP1836, Llavallol, Argentina
[5] Department IFA-Tulln, University of Natural Resources and Life Sciences Vienna (BOKU), Konrad Lorenz Strasse 20, CP 3430, Tulln, Austria
[6] Consejo Nacional de Investigaciones Científicas y Tecnológicas, CABA, Av. Rivadavia 1917, CP 1033, Buenos Aires, Argentina

Received: January 27, 2016
Accepted: June 22, 2016

Abstract: In the present study the aspects of discolouration that could influence both the production and consumption of amaranth were analyzed with the objectives to identify the presence of *Alternaria alternata* on seeds, to analyze possible changes in the anatomy of seed tissues and to detect the presence of fungal secondary metabolites. Component plating, histopathological and mycological analyses on discoloured seeds allowed i) location of propagules of *A. alternata* in all seminal components; ii) observation of hypertrophies in perisperm and embryo and iii) determination of several fungal secondary metabolites, mainly high concentrations of tenuazonic acid. To our knowledge, the information presented in this paper, related to physiological, histopathological changes and fungal secondary metabolites on discoloured seeds of (*Amaranthus mantegazzianus* syn. *A. caudatus* subsp. *mantegazzianus* (Pass) Hanelt affected by *A. alternata*, is the first worldwide record.

Key words: amaranth, fungal secondary metabolites, hypertrophies, seed pathology

Introduction

Amaranth grains were one of the main foods of various indigenous ethnic groups (Aztecs, Incas) that inhabited the Americas in ancient times (NRC 1984). Amaranth considered as nutraceutical crop and known for its nutritional value, has motivated great interest of researchers, producers and consumers in this plant. Currently, amaranth crop has spread to many countries due to its nutritional value and ability to adapt to different agroecological zones where traditional crops fail.

The nutritional characteristic is associated with a high content of proteins, essential amino acids, minerals, vitamins, fats, fibers and starches which are present in the seeds, leaves and stems of the plants (Afolabi *et al.* 1981; Becker *et al*. 1981; Makus and Davis 1984; Willis *et al.* 1984; Teutonico and Knorr 1985).

The seeds of high biological value can carry a diverse mycobiota composed mainly of field and storage fungi, which might damage the seeds and subsequently the crop. *Alternaria alternata* (Fr.) Keissler syn. *A. tenuis* Nees. has been reported as the main contaminant among the field fungi isolated from seeds of amaranth cultivated varieties (Bartolini and Hampton 1989; Noelting *et al*. 2004; Moreno-Velázquez *et al*. 2005; Pusz 2009a; Biasi *et al*. 2013).

Recently, a new disease that affects amaranth seeds, associated mainly with *A. alternata*, was reported in Argentina (Noelting *et al*. 2011). This disease causes a change in the colour of seeds, decreases germination and increases the number of abnormal plants. It is estimated that this pathology may be present in several cultivated areas. However, no data on the discolouration of amaranth seeds by *A. alternata* is available. This scarce information (Noelting *et al*. 2011) may be due to the difficulty in detecting the infection that goes unnoticed to naked eye because of the small size of the seeds (an average diameter 1 mm).

In others crops such as wheat, *A. alternata* is associated with black-point, a worldwide disease affecting wheat grains (Mathur and Cunfer 1993). Symptoms of the black-point include a blackish discolouration around the embryo

*Corresponding address:
mcnoelting@hotmail.com

end (Conner and Davison 1988). The affected grain results in a lower germination rate, and a flour of poor quality with unpleasant taste, and dark color (Lorenz 1986).

Moreover, histological analysis of wheat grains affected by black-point revealed that *A. alternata* is responsible for inducing alterations in embryonic tissues, causing darkening and also loss of cell contents, its hyphae has been observed in all discoloured tissues (Agrawal *et al*. 1987).

As far as amaranth crops are considered, there are no reports related to histological analysis of discoloured seeds infected with *A. alternata*, detected possible alterations in seminal tissues colonized by this microorganism as well as references of possible mycotoxin contamination in amaranth seeds. In this sense it is known that fungal secondary metabolites produced by fungi affect the quality of seeds or grains and their products constituting a potential risk to consumers.

It is known that *Alternaria* metabolites such as alternariol, alternariol monomethyl ether, tenuazonic acid, altenuene and altertoxins have been proved to be harmful to animals (Bottalico and Logrieco 1992). On the other hand, *A. alternata* toxins inhibit the germination of tomato, eggplant, cucumber, okra, rice, mungbean, corn, cotton, peanut and chilli seeds (Theerthagiri *et al*. 2008).

The carried out research aimed at further investigation of discolouration affecting amaranth seeds in relation to location of the *A. alternata* propagules in the seeds, the anatomy of its seminal tissues and type, and concentration of fungal secondary metabolites present.

Materials and Methods

Technique for inducing artificial seed discolouration

In order to induce the completed discolouration of seeds with *A. alternata*, a deep-freeze technique was adapted and modified a difference of the previous technique (Noelting *et al*. 2011). Amaranth seeds with healthy appearance were disinfected in 3% sodium hypochlorite for 5 min, rinsed three times with sterile distilled water and dried between filter papers.

The seeds were plated on Petri dishes containing four layer of filter paper moistened with 5 ml sterile distilled water and incubated first in a growth chamber for 24 h under 12 h photoperiod at 20°C. Then, seeds were transferred to –22°C for 24 h and then seeds were plated on seven-day old *A. alternata* colonies developed on Potato Dextrose Agar (PDA) and incubated at 20°C under 12 h photoperiod for 5 days. Disinfected seeds plated in Petri dishes with PDA and without *A. alternata* colonies were considered as control. Seven days after incubation, the appearance of seeds was evaluated and compared to untreated seeds (control).

Component plating of seeds

Evaluation of seed health conditions allows detecting presence or absence of a pathogen. Both, the blotter method and deep-freezing blotter method are able to reveal *Alternaria* infection (Limonard 1966; Mathur and Kongsdal 2003). We used the component plating technique in order to detect fungal species located in each section of seeds (episperm or seminal coat, perisperm and embryo) on naturally discoloured seeds (S1), artificially discoloured seeds (S2) and normal appearance seeds (S3).

Forty seeds of each sample (S1, S2 and S3) were immersed in water during 10 h, then dissected aseptically with a dissecting needle and separated into three components: seed coat, embryo and perisperm. Each component was surface sterilized for 5 min with 1% sodium hypochlorite, rinsed with sterile distilled water and placed on moisten filter papers placed in polystyrene trays (15 cm wide by 21 cm long). The trays were incubated in a growth chamber at 25±2°C for 7 days and then fungi were identified for each component, and occurrence and incidence of each species were recorded.

Histopathological analysis

These analyses aimed at identifying possible alterations on tissues of discoloured seeds caused by *A. alternata*. Forty seeds from S1, S2 and S3 were first fixed in FAA (formalin-acetic acid-alcohol) and next in paraffin by the traditional method (D'Ambroggio 1986).

Longitudinal and cross sections of seminal tissues (12 μm thick) were cut with a Minot rotary microtome. Tissue sections were stained by staining double method; safranina – fast green (D'Ambroggio 1986) and mounted on slides with a synthetic medium (PMyR) for light microscopy detailed observations.

Mycological analysis

These analyses were carried out to identify presence and validate concentration of fungal secondary metabolites in S1; S2 and S3. Seeds from each group (1 g) were first mixed with 5 ml of extraction solvent acetonitrile/water/acetic acid (79/20/1) and stirred for 10 min followed by ultrasonication for more than 10 min per sample.

Then 100 μl filtered next dried and redissolved in 1,000 μl water-acetonitrile (1/1) and applied on a LC-MS/MS system. The analysis of the fungal secondary metabolites was carried out by using multi-detection method described by Malachova *et al*. 2014.

The same procedure was performed to determine fungal secondary metabolites of the *A. alternata* strain, LPSC N°1085. The strain was grown on Yeast Sucrose Extract agar (YES) at 25°C in darkness for 14 days, following the method described by Samson *et al*. 2002).

The presence of six fungal secondary metabolites, such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), altertoxine I (ALTX-I) and tentoxin (TEN) was determined.

The chromatographic separation was performed at 25°C on a Gemini C18, 150 × 4.6 mm ID, 5 micron particle size, C18 equipped with a 4 mm ID x 3 security guard cartridge (all from Phenomenex, CA, USA, UU) and coupled to a 1290 Series HPLC system (Agilent, Waldbronn, Germany). A QTRAP LC-MS/MS System 5500 (Applied Biosystems, CA, USA) equipped with a Turbo Ion Spray electrospray ionization (ESI) source was used to detect and quantify the fungal metabolites.

Results

Technique for inducing artificial seed discolouration

Seven days after incubation seeds growing on colonies of *A. alternata* showed discolouration. Colour of artificially inoculated seeds was more pronounced (Fig. 1A) in relation to naturally discoloured seeds (Fig. 1B). The non-inoculated seeds showed no signs of discolouration (Fig. 1C) and the presence of *A. alternata* mycelium was observed in their coats.

Component plating technique

Alternaria alternata was the main microorganism detected in episperm, embryo and perisperm of naturally and artificially discoloured seeds and only on coats of normal seeds (Table 1). On the other hand, a lower fungal diversity was detected on normal seeds in relation to naturally and artificially discoloured seeds.

Histopathological analysis

The anatomy and histopathology of artificially and naturally discoloured seeds (Figs. 2A, B) differed from that normal looking seeds that showed no changes in the anatomy of their tissues (Fig. 2C).

Naturally and artificially discoloured seeds showed tissue abnormaties as hypertrophies in perisperm (p) and in the embryo tissues: radicle (r) and cotyledons (co) (Figs. 2A, B).

Ecto- and endophytic hyphae of brown to dark brown colour were observed in seed coat and periperm of seeds with normal appearance and discoloured ones.

Mycological analysis

Artificially and naturally discoloured seeds displayed a higher concentration of fungal secondary metabolites in relation to normal appearance seeds (Table 2). Tenuazonic acid (TeA) was the main mycotoxin detected at high concentrations on discolourated seeds. The other important fungal secondary metabolites detected in order were: AME and AOH.

Fig. 1. Artificially (A) and naturally (B) discoloured amaranth seeds affected by *Alternaria alternata*; normal in appearance seeds (C)

Table 1. Fungal microorganisms isolated from seed tissues: naturally discoloured seeds (S1); artificially discoloured seeds (S2) and normal seeds (S3)

Fungi microorganisms	Seminal tissues								
	episperm			perisperm			embryo		
	S1	S2	S3	S1	S2	S3	S1	S2	S3
Alternaria alternata	7.84	10.00	1.00	5.00	6.00	0.50	2.50	3.00	0.00
Chaetomium globosum	0.00	0.00	1.60	0.00	0.00	0.00	0.00	0.00	0.00
Cladosporium cladosporioides	0.00	0.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00
Epicoccum nigrum	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
Fusarium oxysporum	2.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mucor sp.	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00
Non identified	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00

*values are the average percentage incidence of each isolated microorganism

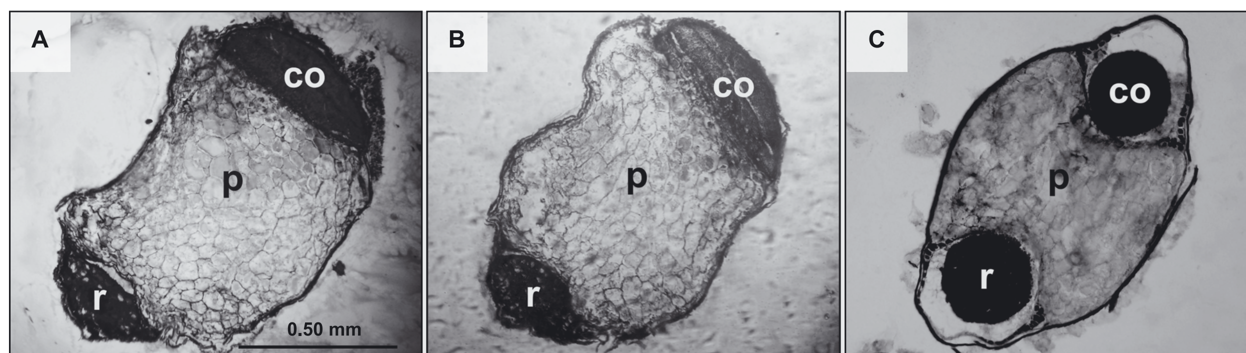

Fig. 2. Hystological sections of artificially discoloured seed (A) and naturally discoloured seed (B) both affected by *Alternaria alternata*, normal in appearance seed (C): radicle (r), cotyledons (co), perisperm (p)

Table 2. Fungal secondary metabolites: AOH – alternariol; AME – alternariol monomethyl ether; TEA – tenuazonic acid; ALT – altenuene; ALTX-1 – altertoxine 1; TEN – tentoxin; LOD – limit of detection; detected on M1 – normal seeds; M2 – naturally discoloured seeds; M3 – artificially discoloured seeds and LPSC N°1085 – strain of *Alternaria alternata*

Samples	*Alternaria* metabolites					
	AOH	AME	TeA	ALT	ALTX1	TEN
M1*	0.001	0.006	1.23	< LOD	< LOD	0.093
M2*	0.923	16.88	4.66	0.03	< LOD	0.430
M3*	128,000	95,000	3,902,000	33,000	9,400	94.03
LPSC N°1085**	154.8	103.45	1155.5	69.45	2.34	< LOD

*values of mycotoxin concentration [μg · g^{-1} of seeds]
**values of mycotoxin concentration [μg · g^{-1} of media]

Discussion

The inoculation of normal seeds with *A. alternata* allowed us: i) to reproduce the discolouration of their coats and the hypertrophies of the perisperm and embryo, phenomenon observed on naturally infected seeds and ii) to identify four similar metabolites detected on natural and artificially discoloured seeds and an strain of *A. alternata* (LPSC N°1085) used in order to induce artificial seed discolouration.

The deep-freezing technique appeared more suitable as compared to previous method (Noelting *et al*. 2011) for induce the discolouration symptoms in amaranth seeds. This technique was positive since all seeds inoculated with *A. alternata* showed change of colour seed discolouration was more intense compared with the conidial suspension inoculation technique used in a previous report (Noelting *et al*. 2011).

On the other hand, the histopathological analysis and plating components allow us to observe the presence of propagules of *A. alternata* in all discoloured seed tissues. These results are consistent with those reported by Agrawal *et al*. (1987) and Őzer (2005) for wheat grains affected by black-point. Moreover, plating components technique can locate the presence of propagules (mycelium and conidia) of *A. alternata* in different tissues of seeds.

The observed hypertrophies in the embryo and perisperm would be associated with a more severe infection by *A. alternata* and also various fungal secondary metabolites might be involved. According to Knoche and Duvick (1987) most of the toxins produced by *Alternaria* have low molecular weight and can cause physiological and pathological changes in the host.

Therefore, it is possible that the morphological changes observed in the embryo and perisperm of discoloured seeds may have been the result of the action of fungal secondary metabolites.

Besides, the presence of *A. alternata* hyphae on the perisperm and embryo of discolourated seeds indicates the infectious nature of this microorganism. Moreover, a higher concentration of *A. alternata* conidia next to the hilar region of amaranth seeds inoculated with the fungus was observed. This fact may indicate that the hilar region is the most vulnerable site for penetration and subsequent colonization of seminal tissues by *A. alternata*. Thus, we can infer that the infection of *A. alternata* could take place either in the early stages of seed development or post seed formation. These results indicate that *A. alternata* is a contaminant and infectious fungus, hence the epidemiological importance of seeds as a source of inoculum of this species as a causal agent of spots and blights on leaves of amaranth plants needs to be recognized (Sánchez-Enciso *et al*. 1990; Noelting *et al*. 2004; Pusz 2009b).

In relation to mycological analysis of seeds, we observed that the type and concentrations of *Alternaria* fungal secondary metabolites detected on normal appearance seeds in the present work are within the same range to those mentioned by Biasi *et al*. 2013. However, the data of discoloured seeds could not be confirmed due to the absence of previous reports in this type of seeds.

The higher concentration of TeA on artificially discoloured seeds could be associated with the used inoculation technique, which favored the rapid colonization of seeds by the pathogen. This mycotoxin is considered as a non-host-specific phytotoxin produced by several fungi including *A. alternata* among others (Montemurro and Visconti 1992). Currently there are no legal limits or criteria set for fungal secondary metabolites produced by *Alternaria* as is was stated by European Food Safety Authority (EFSA) that the contamination of natural products by this organism are not important (EFSA 2011). However, in this study, the detection of high concentrations of fungal secondary metabolites in naturally and artificially discoloured seeds indicates a potential risk for consumers.

For this reason we believe it is advisable to consider strategies inherent to crop management and storage of production in order to reduce the level of fungal secondary metabolites in seeds.

Conclusions

The results found in this work indicate that *A. alternata* is a contaminant and infectious fungus species that is capable to alter seminal tissues and to produce secondary metabolites, which may affect quality and food security of amaranth seeds.

To our knowledge, the information presented in this paper, related to physiological, histopathological changes and fungal secondary metabolites on discoloured seeds of *Amaranthus mantegazzianus* syn. *A. caudatus* subsp. *mantegazzianus* (Pass.) Hanelt affected by *A. alternata*, is the first worldwide record.

Aknowledgements

We acknowledge the Universidad Nacional de La Plata for financial assistance in this study.

The LC-MS/MS system was funded by the Federal Country Lower Austria (grant number: Technopol 085) and co-financed by the European regional development fund of the European Union.

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