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

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**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 agro-ecological 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.
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 Alternaria 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 Alternaria, 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, Alternaria toxins inhibit the germination of tomato, eggplant, cucumber, okra, rice, mungbean, corn, cotton, peanut and chili seeds (Theerthagiri et al. 2008).

The carried out research aimed at further investigation of discoloration affecting amaranth seeds in relation to location of the Alternaria 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 discoloration of seeds with Alternaria, 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 Alternaria 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 Alternaria 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 Alternaria. 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 microscope. 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 Alternaria 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 discoloration

Seven days after incubation seeds growing on colonies of *A. alternata* showed discoloration. 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 discoloration (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 abnormalities 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 perisperm 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 discoloured seeds. The other important fungal secondary metabolites detected in order were: AME and AOH.

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

<table>
<thead>
<tr>
<th>Fungi microorganisms</th>
<th>Seminal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>episperm</td>
</tr>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td><em>Alternaria alternata</em></td>
<td>7.84</td>
</tr>
<tr>
<td><em>Chaetomium globosum</em></td>
<td>0.00</td>
</tr>
<tr>
<td><em>Cladosporium cladosporioides</em></td>
<td>0.00</td>
</tr>
<tr>
<td><em>Epicoccum nigrum</em></td>
<td>0.00</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>2.64</td>
</tr>
<tr>
<td><em>Mucor sp.</em></td>
<td>0.00</td>
</tr>
<tr>
<td>Non identified</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*values are the average percentage incidence of each isolated microorganism*
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 a 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).

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 discoloured 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 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.

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References


