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Research paper

Milk Thistle (*Silybum marianum* (L.) Gaertn.) seed fungi in a sub-tropical district

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Milk thistle, *Silybum marianum* (L.) Gaertn., is known as an edible and medicinal plant applied in traditional medicine, and its seed is used in the production of medicines against liver hyperglycemia. Seed-born fungi may cause plant diseases and produce mycotoxins that are dangerous for animal and human health. Hence, the external and internal fungal infection of milk thistle seeds from six genotypes from six different geographic areas were studied through a deep-freeze blotter test. *Alternaria alternata* was the most prevalent fungus. Other seed-borne fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Rhizopus* were also observed. In addition, highly meaningful differences in the frequency of *A. alternata* were found among seed samples. These findings are not only important in Plant Pathology and Plant Breeding but also in Pharmacy.

Key words: Alternaria, cardus, mycotoxin, seedborne

INTRODUCTION

The medicinal plant, milk thistle (Astraceae: *Silybum marianum* Gaertn.) is currently grown in central Europe, East Asia (China), Australia, North America (the United States), as well as in South America (Das et al. 2008; Machowicz-Stefaniak and Zimowska 2000). The plant seed is the source of flavolignans (Greenlee et al., 2007) applied in medicine production against type II diabetes (Huseini et al., 2006), liver hepatitis viruses and toxins (Fehr, 2007). A narrative review on the clinical efficacy of *S. marianum* seed extract in the treatment of type 2 diabetes mellitus and non-alcoholic fatty liver disease has recently been published, where the anti-diabetic effect of silymarin in four clinical trials in single formula and in one trial in combination with nettle and boswellia gum resin has been reviewed (Mohammadi et al., 2020). Furthermore, the efficacy of silymarin and silybin in the treatment of non-alcoholic fatty liver disease in eleven clinical trials as single formula or in combination with phosphatidylcholine, simvastatin and vitamin E has been reviewed (Mohammadi et al., 2020). The beneficial effect of silymarin is not restricted to its hepatoprotective activity and comprehends anticancer, anti-inflammatory, immunomodulatory, neuroprotective as well as lactogenic effects (Valkova et al., 2020). Out from all plant-based dietary supplements tested, those based on milk thistle were of the highest concentrations of mycotoxins up to 37 mg kg^{-1} Seed (Veprikova et al., 2015). Alternariol-methyl-ether, alternariol, beauvericin, deoxynivalenol, enniatinA, enniatin A1, enniatin B, enniatin B1, HT-2 toxin, T-2 toxin, tentoxin, and zearalenol are known as the most important mycotoxins in milk thistle-based dietary supplements (Pickova et al., 2020).With an eye to the medicinal importance of milk thistle, the present research was conducted (i) to identify potentially important seed-borne fungi of milk thistle, (ii) to identify seed-borne mycotoxigenic fungi on and in the seed of milk thistle, and (iii) to study the fungal infection/ contamination rate of geographically different seed samples which can lead to the clues relevant in plant breeding (Li et al., 2011; Pagán and Garcíá-Arenal, 2018; Villarroel-Zeballos et al., 2012).

MATERIALS AND METHODS

Six seed samples of milk thistle from geographically distinct origins were chosen for this research. The seeds were received from Research Institute of Forests and Rangeland and were gathered after primary

propagation in the research field of Agricultural Sciences and Natural Resources University of Khuzestan made dry and stored inside pockets under room conditions. Primary propagation was made through planting each ecotype on a single line 3 m in length. The distance between two consecutive plants on each line was 30 cm, where the distance between two rows was 50 cm. The seed samples had been named based on their geographical origins: Ardebil, Isfahan, Najaf Abad, Rostam Abad, Shush 1, and Shushtar. These ecotypes except Isfahan, were chosen out from 23 milk thistle ecotypes based on their genetic diversity. DNA-based molecular diversity of all 23 ecotypes had been formerly investigated using a total of 43 ISSR molecular markers, and Ardebil, Najaf Abad, Rostam Abad, Shush (shown as Shush 1 in that study) and Shushtar were chosen as representatives of different groups based on a dendrogram created using 9 ISSR primers (Saghalli et al., 2016).

Isolation and identification of seed-born fungi

The collected seed samples were tested a day after seed samples were harvested. With this study, a deep-freeze blotter test was applied. Abstractly in this method (Rosińska et al., 2013), a given number of each seed were placed on six layers of blotter moistened with distilled water inside Petri dishes. The incubation in the dark at 20°C for 48h was followed by freezing at -20°C for 24h, and incubation at 20°C for ten days when alternating 12h periods of dark and near ultraviolet (NUV) light were used to promote sporulation. This method has been recommended as the most effective method in the detection of the fungi transferred through milk thistle seed (Rosińska et al. 2013), however, the method was applied with some modifications. The seed of each accession was studied in two forms, non-sterilized seeds (to identify internally and externally transferrable seed-borne fungi), and superficially sterilized seeds (to identify internally transferrable seed-borne fungi). The method of sterilization included two steps: superficial sterilization with sodium hypochlorite aqueous solution (1.7%) for 1 minute, followed by the second step of sterilization with an aqueous solution of ethanol (70%) for 1 minute; Both steps were performed under sterile conditions of a biological hood.The final incubation period was considered only 240 hours, 168h inside the incubator at 20°C in dark, followed by 92h incubation under natural light of room conditions in order to induce fungal sporulation (Each Petri dish was placed in a transparent polyethylene bag where a wetted cotton plug had been put to preserve the required humidity keeping sterility conditions). Further extension of the incubation period did not affect the results. Each treatment only included two repeats, in total of 100 seeds for each sample. Tukey's studentized (HSD) test was applied to compare the effect seed sample on the frequency of seed contamination to the fungus *A. alternata*. Two parameters were considered with internal infection of seed samples. The average percentage of internal seed infection to a particular fungus calculated for each seed sample (parameter A).The percentage of the seed samples internally infected to a particular fungus (parameter B).The experiment was performed based on a completely randomized design (CRD) and the data were directly applied in ANOVA analysis with no need to transformation using Bliss formula (Rosińska et al., 2013). Fungal isolates were observed under a microscope (Olympus) and identified using the morphological keys in Watanabe (2002), however, only the most frequently found fungus was identified at species level..

RESULTS AND DISCUSSION

Seed-borne transmission of pathogenic fungi is of high importance in agriculture as well as in human health. Seed-borne fungal infections can lead to seed deterioration, failure in seed germination, seedling pre-emergence/ and post-emergence damping-off diseases. Additionally, seed-borne pathogens can widely extend their geographic distribution (Agrios, 2005). Milk thistle is propagated by sowing its seed, therefore, seed-borne diseases could be of high importance in commercial production of the plant. Using deep-freeze blotter test, potentially seed-born fungi were isolated from milk thistle seeds, *Alternaria alternata*, *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., and *Rhizopus* sp. With superficially sterilized seeds, only *Alternaria alternata*, *Aspergillus* sp. and *Penicillium* sp. were identified as the fungi of internal seed-borne transmission potential. The frequency of the isolation of fungi from the studied seed samples have been exhibited (Table 1).

Table 1. The diversity and frequency of the fungi isolated from the superficially sterilized seed samples of geographically distinct milk thistle (*Silybum marianum*) seed samples

Seed Sample	**Ardebil**	**Isfahan**	**Najaf Abad**	**Rostam Abad**	**Shush 1**	**Shushtar**
Fungus						
Alternaria alternata	0%	4%	0%	20%	0%	0%
Aspergillus sp.	0%	0%	0%	2%	0%	0%
Penicillium sp.	0%	0%	0%	0%	0%	4%

With non-sterilized seeds, *Alternaria alternata*, *Aspergillus* sp., *Fusarium* sp., and *Rhizopus* sp. were isolated. Only seed sample Shushtar (4%) was contaminated with *Fusarium* sp. With the seed contamination to *Aspergillus* sp., the seed sample of Rostam Abad was of the highest rate of contamination (100%), followed with that of Shushtar (12%), and Ardebil (4%). In agreement with former investigations (Machowicz-Stefaniak and Zimowska 2000; Rosińska et al. 2013), *A. alternata* was found as the most prevailed fungus isolated from all non-sterilized seed samples studied a year past harvest.

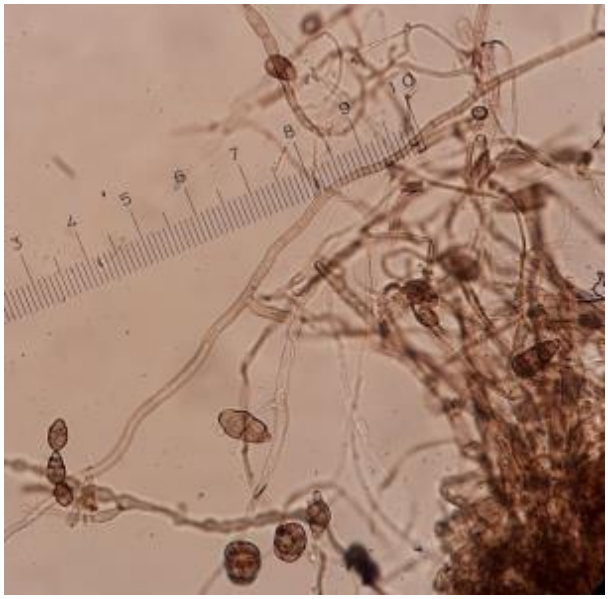

Figure 1. Morphology of *Alternaria alternata* isolated from milk thistle seed (x 400)

Aspergillus sp. was of high abundance only with some seed samples and other fungi were almost absent. Other seed samples were free of *Aspergillus* contamination. Only Shush 1 seed sample was contaminated with *Rhizopus* sp. (2%). All seed samples were contaminated with *A. alternata* (Figure 1), therefore, it was possible to statistically analyze the behavior of seed samples from the viewpoint of their relative rate of

superficial infection with the fungus. Statistical analysis of variance of data made with SAS software (SAS 2009) indicated significant differences in the rate of contamination to *A. alternata* among various seed samples (F5, 6 = 43.80**; CV = 11.58%), Comparison of average contamination rates of the seed samples using Tukey's studentized (HSD) test (α= 0.01) indicated that the seed samples were grouped in two Tukey groups. The samples from Isfahan, Shush 1, and Shushtar which were of average contamination rate of 84%, 96%, and 82%, respectively. The samples from Ardebil, Najaf Abad, and Rostam Abad which were of average contamination rate of 38%, 20%, and 34%, respectively.The highest rate of seed contamination to *A. alternata* was found with the seed sample from Shush 1 (96%), while the least rate was found with that from Najaf Abad (20%).Considering parameter A for *A. alternata*, the highest values were calculated for seed sample from Rostam Abad (20%), followed by that from Isfahan (4%). Other seed samples were free of internal infection. The same parameter was 0.00% with all seed samples when the calculations were made for *Fusarium* sp. and *Rhizopus* sp. With *Aspergillus* and *Penicillium* species, the parameter A values were 0.00% for all seed samples except for the seed sample from Rostam Abad that was internally infected to *Aspergillus* sp. (2%), and that from Shush 1 with internal infection to *Penicillium* sp. (4%).Considering parameter B, the highest rate was obtained with *A. alternata* (33.3%), followed by *Aspergillus* (16.6%) and *Penicillium* (16.6%) species. The parameter was the least for *Fusarium* (0.0%) and *Rhizopus* (0.0%). *A. alternata*, *Aspergillus* spp., and *Penicillium* spp. are airborne molds widely distributed throughout the world, therefore, the differences in the rates of contamination and infection may reflect potential capabilities of milk thistle accessions investigated in this study. In addition to the importance of the detected fungi in plant health and agriculture, most of the detected fungi are known as mycotoxigenic fungi. *A. alternata* as the most common species of the genus is a facultative parasite that can infect a vast range of plants and induce diseases known as Alternaria leaf blights, Alternaria leaf spots, or alternarioses. Also, its prevalence in seed samples studied a year past harvest indicates its importance as a storage fungus. Different temperatures have been reported for growth of the fungus as the minimum ranging from -5 to 6.5ºC, the optimum ranging between 25 and 28ºC, and the maximum ranging from 32 to near 36ºC (Hasija, 1970; Pitt and Hocking, 1997). Also, the fungus can grow at the water activity (aw) down to 0.88 at 25ºC (Hocking et al. 1994). The fungus can grow in the pH range of 2.7-8.0 where the optimal growth is exhibited at pH 4-5.4 (Hasija, 1970). *A. alternata* can grow in oxygen concentrations as low as 0.25% (V/V) in N2, however, its growth rate proportionally changes in an oxygen concentration-dependent manner (Wells and Uota, 1970). These are worrisome facts and indicate the high potential of this fungus as a storage fungus able to impose direct and indirect damages even under storage conditions of controlled atmosphere and cold temperatures.

The fungus produces and secrets three structurally different classes of mycotoxins: dibenzopyrone derivatives (such as alternariol, alternariol monomethyl ether, and altenuene), perylene derivatives (altertoxins I, II, and III), as well as tetrameric acid derivatives (tenuazonic acid), and other less toxic metabolites (Logrieco et al., 2009). Tenuazonic acid is toxic to a number of animals such as chickens, dogs, and mice. However, other mycotoxins, alternariol, alternariol monomethyl ether, altenuene, and altertoxin I are not of high acute toxicities. Alternariol and alternariol monomethyl ether have been reported as mutagenic and genotoxic compounds by several authors (Ostry, 2008). In addition, alternariol interferes in the bioactivity of the enzymes topoisomerase I and II, and may be involved in the impairment of DNA integrity in human colon carcinoma cells (Fehr, 2007). The important role of *A. alternata* in the etiology of human oesophageal cancer has been concluded (Liu et al., 1992).

Aspergillus species produce important mycotoxins such as aflatoxins, sterigmatocystin, ochratoxins, fumonisins, patulin, gliotoxin, and cyclopiazonic acid (Varga et al., 2015). Ochratoxins, also produced by some *Penicillium* spp. (Cabañes et al., 2010), are of proven nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties (Varga et al., 2001). Aflatoxins are identified as one of the major causes of liver cancer (IARC, 2002). The occurrence of aflatoxins in milk thistle herbal supplements has been reported (Tournas et al., 2012). Patulins are also produced by *Penicillium* spp. (Rharmitt et al. 2016), and are no longer known as carcinogenic (Boussabbeh et al., 2016).

Aspergillus and *Penicillium* species are considered as storage fungi of less importance in the field (Agarwal and Sinclair, 1987). In contrast, *Fusarium* species are considered as field fungi of less importance under storage conditions (Maude, 1996; Rosińska et al., 2013). However, *A. alternata* is a fungus of the wide pathogen-host window, that affects all plant developmental stages from seed to seed, and can be regarded as a fungus of the highest importance among the mycotoxigenic fungi isolated from milk thistle seed.

Considering the applications of milk thistle seeds in bakery and herbal medicine production and the prevalence of the contaminations to various mycotoxigenic fungi, it would be very important to check milk thistle seeds and products for the possible mycotoxin contaminations in order to protect consumer health.

Considering the different rate of contamination to the most prevalent mycotoxigenic fungus encountered in milk thistle seed samples, it was revealed that seeds with different backgrounds are not equally prone to seed contamination to *A. alternata*. The difference observed in the behavior of seed samples maybe because of the morphological, biochemical, and physiological characteristics of the maternal plants. Such local accessions would be of importance in breeding for domestic cultivars that escape seed contamination or even more, exhibit resistance to seed infection. Our research led to the following conclusions: (i) the deep-freeze blotter test was found as a cheap, simple, and easy method for the detection of important fungi of milk thistle seed; (ii) *A. alternata* was the most frequently observed fungal species on milk thistle seed propagated in a sub-tropical area in Khuzestan; and (iii) the seed samples from different geographical origins were of highly significant differences in their rate of contamination to *A. alternata*. The seeds from maternal plants in Ardebil, Rostam Abad, and Najaf Abad were of low contamination, however, seeds from Rostam Abad were internally infected to *A. alternata* with an average infection rate of 20%, while other two seed samples did not contain the fungus. Therefore, Ardebil and Najaf Abad samples were considered proper plant materials for breeding programs. The method of defense, whether “active” or “passive’, is immaterial as it can be utilized in practical efforts of plant sanitation, including breeding for any kind of “resistance” (Neergaard, 1977). Resistance to the pathogens (especially mycotoxigenic fungi) of wide pathogen-host window can lead to reduced chemical treatments, less mycotoxin contamination, environmental safety, and further profitability.

AUTHOR CONTRIBUTIONS

Babak Pakdaman Sardrood planned and performed the experiment, identified the fungi, and wrote the article. Mohammad Farkhari prepared the required seed, and analyzed the data.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICS APPROVAL

Not applicable.

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