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Saffron corm as a natural source of fungicides: The role of saponins in the underground

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ABSTRACT

Fungi cause important deteriorations of corms from *Crocus sativus* L. In order to screen the antifungal properties of this organ to fight such infections, two independent experiments based on the lyophilized and sterilized external (peel) and internal parts of the corm were conducted against five fungi isolated from infected corms during August. The minimum inhibitory concentrations (MIC) after 30 days of the peel treatments were 5.4% against *Aspergillus niger*, 3.9% against *Bipolaris spicifera*, *Fusarium oxysporum*, *Penicillium raistriicki* and 2.3% against *Rhizopus nigricans* while the MIC of the internal part were not detected for *A. niger* and *B. spicifera*, 7.0% against *F. oxysporum* and *P. raistriicki* and 3.9% against *R. nigricans*. The higher toxicity of the peel against fungi led us to investigate the influence of the saponins exclusively detected on the external part of the corm, as partially responsible for the extra observed effect. The main influence of these compounds on the toxicity was against *F. oxysporum*, the most desvasting pathogen in saffron corms, followed by *B. spicifera* and *A. niger*. The growth inhibition of *P. raistriicki* and *R. nigricans* was almost negligible. However, other compounds such as phenolics compounds could also be responsible for the fungicidal activity detected. These results illustrate that saffron corms could be further exploited in order to discover new phytochemical products with antifungal properties.

1. Introduction

Crocus sativus, or saffron, is a triploid sterile plant propagated by corms, cultivated only to produce red styles branches. As a subterranean organ, the corms in their natural environment are constantly under siege from a multitude of disease-causing organisms including viruses, nematodes and especially fungi. Surprisingly, pathogenic bacteria have not been identified as responsible for important saffron losses, suggesting the presence of important defence barriers in saffron which prevent bacteria colonization. This ability to resist disease also depends on soil conditions such as structure, compaction, drainage, temperature and level of biological activity, along with cultural practices that influence plant development, such as planting date and application of fertilisers or herbicides (Ahrazem et al., 2010).

The developmental stage of the corm also plays an important role in the success of fungi infections since the secondary metabolites that could act as phytoprotectans change throughout the biological cycle. Corms are collected from May to August, and then undergo a selective process for the next cultivation (Negbi, 1990). The biological cycle of the corms is difficult to understand since there are stages where three corm generations co-exist simultaneously. The apical and sub-apical buds are activated at the end of August when roots appear in the mother corm. During October, the development of the embryonic buds of these stems will culminate in true floral organs. In November, immediately after flowering, the base of the stems that are in contact with the maternal bulb being to swell and generate the new corm (López and Gómez-Gómez, 2009). The accumulation of reserves from green leaves causes the swelling of the daughter or waking corms (Esmaeili et al., 2011), although the mother corm, which has roots throughout this period, also maintains its growth. In April or May the mother corm is completely senescent and the daughter corm becomes independent and

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dormant. From June to August the corm does not undergo changes in its size; it remains dormant in order to survive summer drought and high temperatures (López and Gómez-Gómez, 2009).

In the present study several fungi such as *Aspergillus niger*, *Bipolaris spicifera*, *Fusarium oxysporum*, *Penicillium raistriicki* and *Rhizopus nigricans* were isolated from saffron corms during August. *F. oxysporum* and *B. spicifera* were also isolated from different plant materials. These last two fungi are considered as real pathogens of *C. sativus* although *B. spicifera* commonly occurs on grasses. All the above fungi are common in soil (Ahrazem et al., 2010). The genus *Aspergillus* is a diverse and familiar group of ascomycetes which is mostly saprophytic, even though it includes human pathogens, plant pathogens, and species useful in industrial processes and genetic research. *A. niger* is a fungi found worldwide which is responsible for black mould. It is transmitted by contaminated corms or soil, with the infection usually starting at the root initiation stage and continuing through corm storage (reviewed by Kniemeyer, 2011). *Penicillium* rot, more commonly referred to as blue mould rot, is one of the primary agents responsible for crop losses of flower bulbs and vegetables during storage (Cappelli, 1994). Even though several *Penicillium* species have been isolated from infected saffron corms only *Penicillium vinaceum* has actually been documented (Zheng et al., 2012). Common root rot is caused by *Cochliobolus* or *Bipolaris* species producing symptoms identical to *Fusarium* diseases. Root development is reduced and plants are easy to pull out of the soil. Lower stems, leaf sheaths, and roots have brown lesions resulting in a reduced number of new corms. *Rhizopus* spp. are zygomycetes, a type of primitive fungi, which are either saprophytes or weak parasites of plants where they cause soft rots or moulds. These fungi enter into the plant through wounds present on the surface. *R. nigricans* has been isolated from wounds present in *C. sativus* corms (Ahrazem et al., 2010). The genus *Fusarium* comprises several fungal species widely distributed in soils and organic substrates. One of the most relevant species of this genus is *F. oxysporum*, which causes vascular wilt and root rot in more than 100 species of plants (Berrocal-Lobo and Molina, 2008). *Fusarium* corm rot incited by *F. oxysporum* is the most destructive disease in saffron, having caused severe losses in Italy (Cappelli, 1994). The disease has been referred to by various names, including dry rot, brown rot, basal rot and yellows. The major symptoms of the disease occur during the flowering time when the infected plants show drooping, damping-off, yellowing and wilting of shoots, as well as basal stem rot and corm rot. The pathogen survives in infected corms and in the soil as mycelium, chlamydospores, macroconidia and microconidia (Brayford, 1996). Plants may become infected in the field, when germinating spores or mycelia enter into the roots directly or through wounds. Most probably, the pathogen is introduced into new saffron-growing regions via contaminated corms (Cappelli and Di Minco, 1999). The disease was detected for the first time in Japan (Yamamoto et al., 1954) and was later reported in India (Shah and Srivastava, 1984), Spain (García-Jiménez and Alfaro-García, 1987) and Italy (Cappelli, 1994).

Plants have evolved intricate mechanisms to recognize and defend themselves against the wide array of these disease-causing agents, including fungi. For instance, plants constitutively produce secondary metabolites, many of which can act as antimicrobial compounds for defence against microorganisms (Dixon, 2001). These compounds, such as saponins, phenolic compounds, flavonoids and many others, represent the first chemical barriers to infection and are associated with non-host resistance (Ahrazem et al., 2010). Saponins are glycosylated triterpenoids, steroids, or steroidal alkaloid molecules with antifungal activity which are constitutively produced in many plants and can also be induced as a result of a pathogen infection (Osbourn et al., 1996). Saffron is characterized by the presence of saponins in stigma (Hosseinzadeh and Younesi, 2002) and in corm tissue (Rubio-Moraga et al., 2011) where they seem to play antifungal roles.

The most common way for the control of fungal diseases is using synthetic chemicals as fungicides. However, because of their possible persistence and accumulation in the environment and finally in consumers the public opinion has reservations against chemical fungicides. Furthermore, the application of these synthetic chemicals products over a length of time may induce the appearance of resistant strains (Chang et al., 2008). Natural products, mainly those isolated from plants, provide a significant source of potential drugs which humankind has identified not only for use as phytomedicines and herbal remedies, but also for use in current antibiotics, anticancer drugs, food preservatives and additives for human or animal use, in cosmetics and perfumes, and other industrial fields (Gonçalves et al., 2012). During the last two decades, applications of some natural compounds such as essential oils and extracts for biological control of pathogens have been described (Kalemba and Kunicka, 2003; Martin and Ernst, 2004; Palmeira-de-Oliveira et al., 2009).

The aim of the present study is to research the potential antifungal properties of saffron corm extracts, and to determine the role of saffron corm saponins in defence mechanisms.

2. Materials and methods

2.1. Plant material

Corms from *C. sativus* were collected during August 2009 from farmers in Tarazona de la Mancha (Albacete). A voucher specimen was deposited in the Botanical Garden of Albacete, Castilla La-Mancha University, Albacete, Spain.

2.2. Extraction and isolation of saponins

The external part of 1 kg of corms was lyophilized and extracted 3 times with 50% aqueous isopropanol at room temperature. The extract was concentrated and the oily residue was re-dissolved in 30% aqueous methanol and precipitated with acetone (1:1). After centrifuging, the pellet was re-dissolved three times in pure methanol and precipitated with acetone (1:1) to obtain a crude mixture of saponins named Csap. This was dissolved in 50% aqueous acetonitrile and fractionated on a reversed-phase HPLC (Konik 560, Spain) using a Cosmosil 5 C_{18}-AR II column (4.6 × 250 mm, Hewlett Packard, Palo Alto, CA). The column was equilibrated with 36% acetonitrile (v/v), containing 0.05% trifluoroacetic acid (v/v), and eluted with the following acetonitrile gradient containing 0.05% trifluoroacetic acid (v/v): from 36 to 44% in 15 min, from 44 to 82% in 1 min, 82% for 5 min, from 82 to 36% in 1 min, and 5 min to re-equilibrate the column. The flow rate was 1.5 mL/min and the detection wavelength was 208 nm. The saponins fraction (CS5) was obtained from 10 to 15 min (Rubio-Moraga et al., 2011).

2.3. Determination of total phenolic and flavonoid contents

Total phenolic contents of saffron corms were determined by the Folin–Ciocalteu method (Meda et al., 2005). Aliquots of 0.5 g of internal and external fresh corms collected in August were dissolved in 1 mL of deionized water. After shaking 10 min at 200 rpm at room temperature, the aliquots were centrifuged at 8000 rpm for 20 min. The supernatant (0.1 mL) was mixed with 2.8 mL of deionized water, 2 mL of 2% sodium carbonate (Na_2CO_3), and 0.1 mL of 50% Folin–Ciocalteau reagent. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm against a deionized water blank on a spectrophotometer (Cary 60 UV-Vis,