ACCUMULATION OF SOLUBLE PHENOLIC COMPOUNDS IN SUNFLOWER CAPITULA CORRELATES WITH RESISTANCE TO *SCLEROTINIA SCLEROTIORUM*

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Summary

Disease symptoms and total soluble phenolics content have been analysed in four sunflower (*Helianthus annuus* L.) lines with different resistance levels (from highly susceptible to resistant) to head rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary.

At the beginning of the flowering stage, capitula were inoculated by spraying with a water suspension of ascospores, and disease symptoms were evaluated from day 6 to day 14 after inoculation. The most susceptible genotypes showed all their ovaries to be necrosed and abundant lesions in corollas, bracts and receptacle. In the resistant line, the ovary and corolla werer only partially necrosed with no symptoms in the bracts or the receptacle.

Total soluble phenolics were extracted and quantified from different parts of the capitulum in both inoculated and non-inoculated plants. The amount of phenolic compounds depended on the sunflower line, the time after inoculation, and the tissue. Higher constitutive and induced phenolic content as well as phenylalanine ammonia-lyase activity were present in the most resistant line, these differences being correlated with the absence/presence of disease symptoms.

Abbreviations: BSA, bovine seroalbumine; PAL , phenylalanine ammonia-lyase; PMSF, phenylmethylsulfonyl fluoride; PVPP, polyvinylpolypyrrolidone; TLC, thin layer chromatography.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a worldwide distributed sunflower pathogen attacking most plant parts, including root, stem, capitulum, leaf and terminal bud at any developmental stage. Under favourable climatic conditions the fungus causes important yield reductions, especially in the case of stem (stalk rot) or capitulum (head rot) infection (Purdy, 1979). As chemical control is not practical, genetic control through the development of resistant lines appears to be the best crop protection strategy. Immunity to the fungus has not yet been found, although a wide range of susceptibility to attacks under field conditions has been described among sunflower inbred lines, varieties and hybrids (Gulya, 1985; Bazzalo et al., 1991). Accordingly, a major goal of research is to identify compounds involved in resistance, in order to be used as molecular markers in plant breeding programmes or to design appropriate control strategies.

Phenols have long been associated with passive (preinfectional) and active (postinfectional) defence responses of plants against a number of pests and pathogens (Nicholson and Hammerschmidt, 1992). Some previous biochemical

studies of the *Sclerotinia*-sunflower interaction directed towars evaluating soluble phenolics in infected- and non-infected crop lines and wild relatives have been reported (Bazzalo et al., 1985, 1987; Hemery-Tardin et al., 1998). These experiments have been performed under controlled conditions and were mainly focused on vegetative tissue, despite the fact of the devastating consequences of the infection at the closed flower-bud developmental stage (Bazzalo et al., 1985). The accumulation of soluble phenolic compounds, their deposition on cell walls, melanization and lignification are typical sunflower responses to *Sclerotinia* infection which takes place in stem and leaves in both infected and healthy surrounding tissue, this pathogen-induced accumulation being, in general, lower in susceptible *Sclerotinia* varieties (Bazzalo et al., 1985, 1987; Hemery-Tardin et al., 1998). In a previous paper, Bazzalo et al. (1991), present data of relationships between the field reactions of eight sunflower-inbred lines to the basal stalk rot and phenolic concentration in healthy tissue around the lesion zones. A strong negative correlation between postinfectional, but not preinfectional, phenolic accumulation and the wilting range (stalk rot) for individual plants was obtained, although a clear interpretation of these results in terms of genetic resistance is difficult due to the great influence of environmental factors on the production of phenolic compounds (Jorrín & Prats, 1999).

The aim of this work was: a) to evaluate different sunflower genotypes for their resistance/susceptibility to Sclerotinia head rot; b) to test if there is any

relationship between symptomatology and the level of pre- and postinfectional total soluble phenolics and phenylalanine ammonia-lyase activity in capitula.

MATERIAL AND METHODS

Plant and fungal material

The sunflower lines 20303, 30154, FRTS and 1V8410 used in this study were provided by Advanta Seeds S.A.I.C. (Balcarce, Argentina). These genotypes have been previously selected on the basis of their different response to *Sclerotinia* under field conditions (unpublished results). Seeds were sown in 20 litre pots containing a methyl bromide sterilised peat:perlite (3:1) mixture. Plants grew in a greenhouse with a 20ºC average temperature and 80% HR.

Inocula source was initially obtained from natural infected plants at balcarce production field in 1991. Every year this population is employed to artificially inoculate different sunflower germplasm in the field by ascosporic method. Sclerotia obtained from approximately 5000 inoculated plants were bulked and induced to carpogenic germination (15 days at -20ºC treatment). Then 30 trays with 20-30 sclerotia were incubated in a humid chamber on wet soil with a 13 h photoperiod (60μE m^2s^{-1}) and 18ºC temperature. After a month ascospores were harvested and stored from apothecia and stored at -20ºC and dry conditions until use. From each tray 5-8 Petri dished with 10-20 apothecia were obtained.

Ascospores obtained from Petri dished randomly choose were employed for inoculation and bioassays. Observation of incubated ascospores on control media (without plant extracts) indicated a high level of viability (approximately 100%)

Inoculation and sample collection

At the beginning of the flowering stage, capitula were inoculated by spraying with a 1.5 ml of a water suspension of ascospores (20 000 ascospores ml^{-1}), with control plants only receiving water treatment. In order to ensure high humidity conditions with no thermal stress, plants were covered with a paper bag. Capitula were visually observed during 14 days and samples collected 6, 10 and 14 days after inoculation. Inner bracts and corollas from the external rays were separated, the necrosed zones removed and healthy surrounding tissue frozen in liquid nitrogen and stored at -70ºC until biochemical analysis. Similar plant tissue was obtained from non-inoculated controls at the same moment. A factorial experiment was performed with an incomplete block design with 8 replicates. The three factors were: sunflower line, time after beginning of flowering and inoculation.

For seed analysis, the hull and kernel were separated. Leaves from 20, 40 and 60 days old were removed and frozen in liquid nitrogen and stored at -70ºC until biochemical analysis. The experiment was performed with a complete block design with 8 replicates.

Disease symptoms evaluation

Resistance/susceptibility was evaluated either visually or with a microscope by observing the presence of mycelium in the anthers and necrotic lesions in different parts of the capitulum (receptacle, bracts and corollas). Successful ascospore germination is indicated as percentage of plants showing mycelium on the anthers. Disease incidence is indicated as the percentage of plants showing visible lesions. Necrotic lesions in corollas, bracts and receptacle were quantified by using a discontinuous scale from 0 (no necrotic lesions observed) to 100% (tissue totally necrosed) with intermediate values of 10, 25, 50 and 75 indicating the percentage of the total of corollas, bracts or receptacle affected.

Biochemical analysis

For phenolic determination, tissue samples were extracted as reported in Prats et al. (2002). Frozen tissue (leaves, bracts and corollas) was homogenised with prechilled (-20^0C) 1:10 (w:v) acetone in a mortar. After filtering off the solvent, the residue was further sequentially extracted with a similar volume of acetone and acetone:methanol (1:1 v/v). The combined organic extracts were concentrated under vacuum, redissolved in a minimum volume of methanol and then analysed both spectrophotometrically and by TLC.

The seed analysis was achieved according to Pedrosa *et al.,* (2000) with some modifications. A sample of 200 mg of sunflower hull or kernel was pulverised with liquid nitrogen and suspended in 2 ml 80% ethanol, keeping the mixture shaking mecanically for 30 min. The suspension was centrifuged at 12000g for 10 minutes and the supernatant collected. The pellet was extracted 3 more times, the supernatants combined and the ethanol evaporated under vacuum. The volume of the aqueous phase was adjusted to 2 ml and partitioned firstly against petroleum ether to eliminate oil and pigments and secondly against ethyl acetate (three times, 2 ml each) to recover the phenolics. The ethyl acetate solution was vacuum dried and the residue redissolved in a minimum volume of methanol.

Total phenolics were determined by using the Folin-Ciocalteu reagent, as previously reported (López Valbuena, 1980; Prats, 2002). TLC analysis of the methanolic extract was performed according to Gutiérrez-Mellado et al. (1996), by using Silicagel 60 F_{254} plates (Merck) and ethylacetate as the mobile phase. Plates were visualised under UV light (254 nm) lamp.

For PAL extraction and assay, frozen bracts were homogenised in liquid nitrogen and the powder extracted with 50 mM Tris-HCl buffer (pH 8.5) containing 1 mM PMSF, 14 mM 2-Mercaptoethanol and 5% insoluble PVPP. The extract was centrifuged at 10000g for 15 minutes and the supernatant desalted with NAP 5 columns (Pharmacia). PAL activity was assayed as described by Jorrin & Dixon (1990).

Protein content was determined using the Bradford reagent (Sigma) with BSA as the protein standard as recommended by the manufacturer.

Sclerotinia growth bioassay

A quantitative test for *Sclerotinia* growth inhibition was performed in flat-bottomed microtitre plates. Corollas and bracts methanolic extracts (obtained from 1 gram of tissue 14 days after inoculation) were vacuum dried and the residue redissolved in 100µl of CZAPEK-DOX BROTH (Sigma) medium containing ascospores (20000 ml^{-1}). The plates were incubated for 18 hours at 21 ºC with continuous illumination (4µEm^2s^{-1}). The length of the ascospore germinative tube was measured with an inverted white light microscope (CK2 Olympus, 100 x).

RESULTS

Evaluation of resistance/susceptibility

Disease symptoms were evaluated when they started to be visible (6 days after inoculation) and observations followed until 14 days' postinoculation when the tested genotypes showed big differences. At day 14, the most susceptible plants presented an ample number of lesions in all the capitula, being watery, pale brown and with no defined boundaries. On the contrary , most resistant plants lesions were

much smaller, dry, dark brown and clearly restricted to the ovary and corolla. These tissues were only partially necrosed, with no symptoms in the bracts or the receptacle (Figure 1).

The presence of mycelium on the anthers was observed in all the lines, indicating successful germination of some ascospore independently of the plant genotype, although there were small quantitative differences between them as refers to the number of plants presenting mycelium at the different days post-inoculation and the amount of mycelium, estimated visually (Table 1). Nevertheless inhibition of certain number of ascospores could not been excluded although the germination bioassays showed no significant differences related to germination between genotypes (Table2). A portion of less virulent ascospores could have been inhibited and did not germinate.

The differences between lines corresponded to the number of plants showing visible lesions (incidence) and to the level of the fungal invasion of the different organs (Table 1). Thus, at day 14, the most susceptible genotype (20303) showed all the ovaries necrosed and abundant lesions in corollas, bracts and receptacle, with 87.5 of the plants showing visible tissue damage. Conversely, the highly resistant line (1V8410) showed only partially necrosed ovaries and corollas with no symptoms in bracts or receptacle and with only 28.5 of the plants presenting visible lesions. Those of intermediate resistance (30154 and FRTS), showed values of incidence of, respectively, 87.5 and 62.5 %, a high invasion of the ovaries and a

partial one of the corollas with necroses in bracts and receptacle being, or not, present.

Phenolic content and PAL activity

Total soluble phenolic content was determined in corollas and bracts of non-inoculated (constitutive levels) and inoculated (induced levels) plants. Data corresponding to corolla and bract extracts are presented in Figure 2. Higher constitutive levels were detected in corollas than in bracts in all cases, as expected for cells rich in flavonoid pigments. In bracts, significant differences in soluble phenolics at days 10 and 14 were found between the most resistant 1V8410 genotype and the other, specially the most susceptible (Tukey $p<0.001$), with the highest value corresponding to, which presented an evident accumulation of total phenolics along the time (Figure 2A).

As a result of the inoculation, an increase in the content of phenolic compounds in bracts but not in corollas and only in the most resistant plants (1V8410) (Tukey $p<0.05$) was observed.

A similar tendency as described for phenolic content was observed for PAL activity (Figure 3). Thus, 1V8410 bract extracts showed higher PAL activity values than the 20303 at 6, and 10 days after flowering, these differences being statistically significant at day 10 (Tukey $p<0.01$). The inoculation of plants caused

an increase in PAL activity in both genotypes, being transient in the resistant one, which showed maximum values at day 10 and ulterior decay at day 14.

The analysis of extracts by TLC revealed the existence of changes in the phenolic profile, both qualitative and quantitative, and between genotypes as well as between inoculated and non-inoculated plants. Some compounds were only present in 1V8410 extracts and were not detected, or were at very low concentrations, in susceptible lines. By comparison with proper standards, some of these compounds were identified as the 7-hydroxylated simple coumarins scopoletin and ayapin (Figure 4).

The analysis of phenolics in healthy leaves and bracts of 20303 and 1V8410 plants at different developmental stages showed a progressive accumulation from day 20 to day 74 (14 days after flowering) with higher values in bracts than in leaves and in the resistant than the susceptible genotype (Figure 5). Significant differences (Tukey $p<0.001$) in bracts, but not in leaves, between genotypes were obtained. In seeds, most of the phenolic compounds were accumulated in kernel, with values of 2,13± 0,12 and 1,61± 0,08 mg eq. chlorogenic acid g^{-1} fresh weight for 1V8410 and 20303, respectively. Values for hull were of 0,41±0,07 and 0,23±0,01 mg eq. chlorogenic acid g^{-1} fresh weight for resistant and susceptible genotypes.

Antifungal activity of phenolic extracts

In order to test the presence of antifungal compounds in the phenolic extracts and to compare results obtained from the different genotypes, an *in vitro* assay of *S. sclerotiorum* spore growth in the presence of bract and corollas extracts was performed. Phenolic extracts from the resistant 1V8410 genotypes were highly inhibitory of germination and growth of primary hypha, both in the case of bracts and corollas (almost 100% inhibition) (Figure 6). Percentages of inhibition with extract from the other genotypes ranged from 0% to 35% (Figure 5).

DISCUSSION

Head rot damages are usually visualised in the field when plants are near to physiological maturity. Nevertheless, infection takes place at the beginning of flowering. Ascospore artificial inoculation, in contrast with the mycelia test, permits the detection of the presence of barriers in capitulum parts where infection really occurs and seems to be more efficient than the alternative mycelium test (Castaño et al., 2001).

By inoculating capitula at the beginning of the flowering with *Sclerotinia sclerotiorum* and by following its evolution in different organs (anthers, ovaries, corollas and bracts), both visually and microscopically, the disease resistance levels to head rot in four sunflower genotypes have been evaluated and the

mechanisms of resistance hypothesised.

As a parameter of resistance, the percentage of plants showing visible lesions as well as the presence and size of necrotic lesions in receptacle, bracts and corollas have been used. According to these parameters1V8410 behaved as highly resistant, FRTS as resistant and 30154 and 20303 as susceptible. This early evaluation is in agreement with that previously obtained under field conditions (Bazzalo, unpublished).

The presence of mycelium on the anthers in all the lines indicated the active germination of some ascospores, an observation that has been previously reported by Says-Lesage and Tourvielle (1988), It is possible that a number of the less virulent ascospores were inhibited, however the bioassays with the extracts of the different genotypes showed that ascospores were not significantly inhibited . Respect to other pathosystem, sunflower-*Puccinia helianthi*, resistance associated with the excretion to the leaf surface of fungal spore germination inhibitors has been shown (Prats et al., 2002).

Partial necroses of the corollas and an absence of symptoms in bracts and receptacle in the 1V8410 genotype suggest that fungal invasion from the infection site (anthers) to other part of the capitulum is prevented. This indicates the existence of preformed or induced chemical and/or physical barriers. Anatomical studies of tolerant and susceptible sunflower plants to basal stalk rot revealed that in resistant

cultivars fungal mycelium is restricted to cortical tissue while in susceptible ones it reaches the stem pith causing the collapse of plants (Bazzalo et al., 1987).

It has been very well documented in different pathosystems that phenolic compounds can play an important role in disease resistance, being defined as phytoalexins or phytoanticipins, or acting as a physical barrier (lignins), thus preventing plant tissue colonisation (Nicholson and Hammerschmidt, 1992; Friend, 1977). Thus, increased synthesis and accumulation of phenolic compounds is a typical response of sunflower to fungal infections, including *Plasmopara halstedii* (sunflower downy mildew) and *Puccinia helianthi* (sunflower rust) (López Valbuena, 1980; Prats et al., 2002), as well as to parasitic plants (*Orobanche cernua,* sunflower broomrape; Serghini et al., 2001).

Data presented here indicate that in sunflower, the induced synthesis and accumulation of phenolic compounds could be part of the defensive mechanisms against *Sclerotinia sclerotiorum.* This hypothesis is supported by the following experimental data:

i) Higher accumulation of phenolic compounds in resistant than in susceptible varieties in both inoculated and non-inoculated plants. This difference has been clearly observed in bracts, but not in corollas. It is possible than the higher phenolic content in this organ due to the presence of flavonoid pigments mask differences in minority antifungal

compounds. Our data confirm previously published results on phenolic induction in *Helianthus* wild species and cultivated sunflower vegetative tissue (leaves and stem) and in response to *Sclerotinia* infection (Bazzalo et al., 1985; 1986; 1991; Mondolot-Cosson et al., 1997; Tourvieille et al., 1997).

ii) Higher constitutive as well as inducible levels of extractable PAL activity in bracts of the resistant than in the susceptible variety. PAL catalyzes the first reaction of the phenylpropanoid pathway which provides the basic skeleton to build the different families of phenolics (coumarins, flavonoids, lignins) phenolic derivatives, its activity being correlated with the level of synthesis of the phenolic compound (Bevan *et al.,* 1989). It has been recognized that PAL plays a key regulatory role in phenolic biosynthesis. The induction of PAL activity preceeding an increase in the phenolics content has been observed in response to fungal infection in a number of systems (Tena and López-Valbuena, 1983; Pereira *et al.,* 1999; Mazeyrat *et al.,* 1999), previously studied.

iii) Phenolic extracts from resistant plants showed a more potent inhibitory activity on the *Sclerotinia* ascospores growth than those obtained from susceptible ones.

However this study does not discard the existence of other defence mechanisms. Thus, the de la Canal Research group has described the presence in sunflower flowers of a potent antifungal protein (Giudici et al., 2000). Additive gene

effects have been proven to contribute significantly to resistance in sunflower capitula (Castaño et al., 2001)

A great number of lines would have to be analyzed in order to confirm the correlation between the preinfectional level of phenolics and resistance found in bracts. In previous studies with vegetative tissue of wild *Helianthus* spp. and cultivated sunflower such a correlationship has been found between postinfectional but not preinfectional levels (Bazzalo et al., 1991) or it have been difficult to establish (Tourvieille et al., 1997). These results are very promising as it could be possible to use specific phenolic compounds directly as biochemical markers or through associated molecular markers in plant breeding programmes for resistance to *Sclerotinia*. Nowadays we are working on the identification of specific compounds, which discriminate between resistance and susceptibility better than total phenolics compounds. In this respect, TLC analysis reveals clear differences in the content of coumarins and other unidentified compounds between different behaviour lines. The sunflower coumarins scopoletin and ayapin are potent *Sclerotinia* growth inhibitors (Urdangarin et al., 1999). Work is in progress with respect to coumarin purification and quantitation as well to the characterization of those unidentified compounds.

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TABLES

Table 1. Disease symptoms in *Sclerotinia* infected sunflower plants. Symptoms were evaluated 6, 10 and 14 days after inoculation. Anther infection is indicated as percentage of the plants presenting mycelium in the anthers. Disease incidence is indicated as percentage of plants showing visible lesion. Necrotic lesions in corollas, bracts and receptacle were quantified by using a discontinuous scale from 0 (no necrotic lesions observed) to 100% (tissue totally necrosed) indicating the percentage of the total corollas, bracts or receptacle affected. Values are mean of 8 replicates.

Days after Flowering	Genotypes 20303	30154	FRTS	1V8410
	Anther infection			
6	1.0	12.5	12.5	14.2
10	100.0	57.1	28.5	75.0
14	100.0	100.0	71.4	87.5
	Incidence			
6	0.0	0.0	0.0	0.0
10	50.0	25.0	14.2	14.2
14	87.5	87.5	62.5	28.5
	Necroses in corollas			
6	0.0	5.0	5.0	5.0
10	15.6	36.2	10.0	10.0
14	57.5	32.5	17.0	11.2
	Necroses in bracts			
6	0.0	0.0	0.0	0.0
10	16.0	10.0	10.0	1.0
14	63.5	18.0	5.0	1.0
	Necroses in receptacle			
6	0.0	0.0	0.0	0.0
10	22.6	10.0	2.0	1.0
14	36.4	20.0	1.0	1.0

Table 2: Percentage of ascospores germination in presence of bracts (A) and corollas (B) phenolic extracts. Values are mean of 5 replicates (scoring 50 spores per replication) ± s.e.

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	Genotypes				
	20303	**30154**	**FRTS**	**1V8410**	**Control**
Bracts	98.2 ± 3.2	95.6 ± 3.7	97.6 ± 1.7	97.4 ± 3.6	98.1 ± 1.9
Corollas	99.2 ± 2.1	96.3 ± 5.1	98.2 ± 2.8	98.5 ± 2.2	98.3 ± 2.1

FIGURES

Figure 1. Disease symptoms in capitula, bracts, ovaries and corollas 14 days after inoculation. A:20303; B: 1V8410

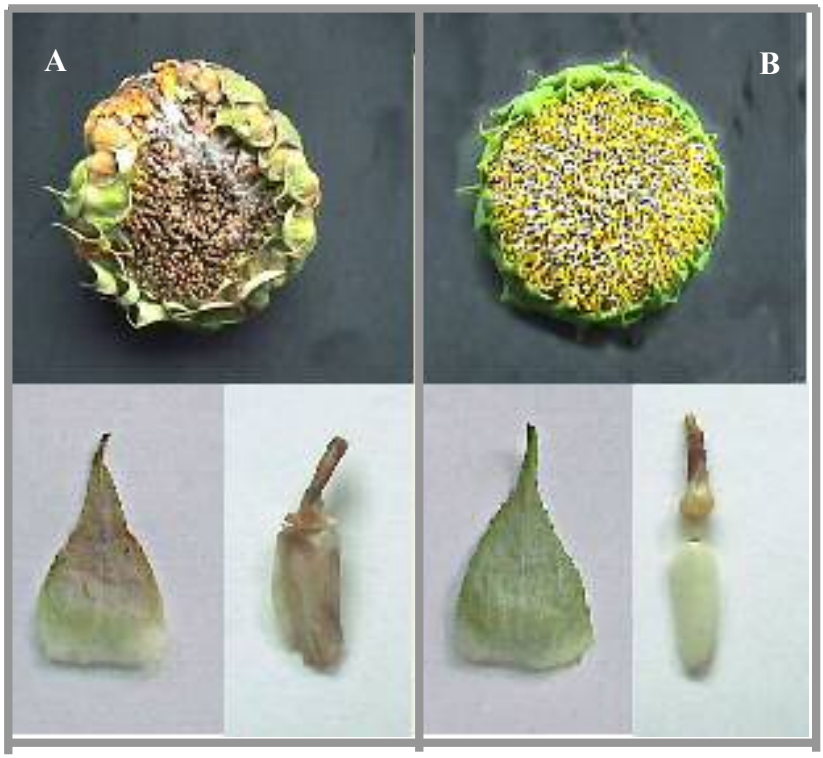

Figure 2. Phenolic content in bracts (A) and corollas (B) of control (non-inoculated, left) and inoculated (right) sunflower capitula. Data correspond to the four lines tested at different times postinoculation (6, 10 and 14 days). Values, expressed as mg equivalent of chlorogenic acid g^{-1} fresh weight, are mean of 8 replicates ± S.E.

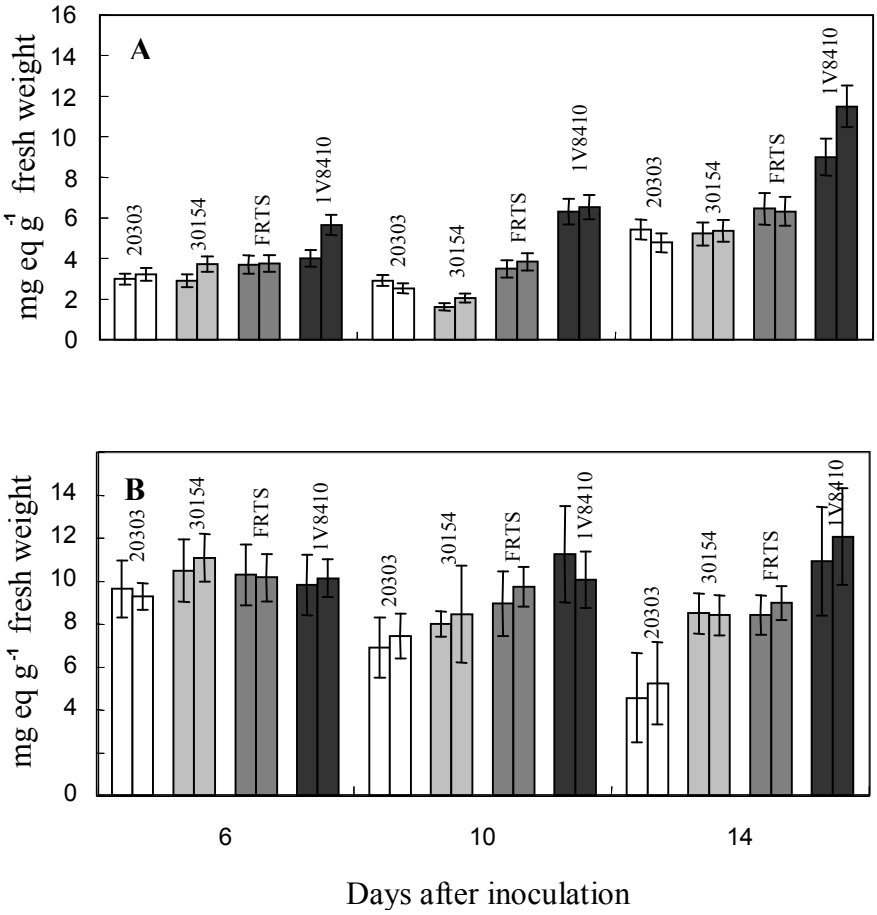

Figure 3: PAL activity in bracts of resistant 1V8410 and susceptible 20303 genotypes in inoculated and non inoculated plants. Values, expressed as nKat mg^{-1} protein, are mean of 11 replicates ± s.e 1V8410 (▲ ,△); 20303 (● ,○); inoculated (▲ , ●); non inoculated (△ , ○)

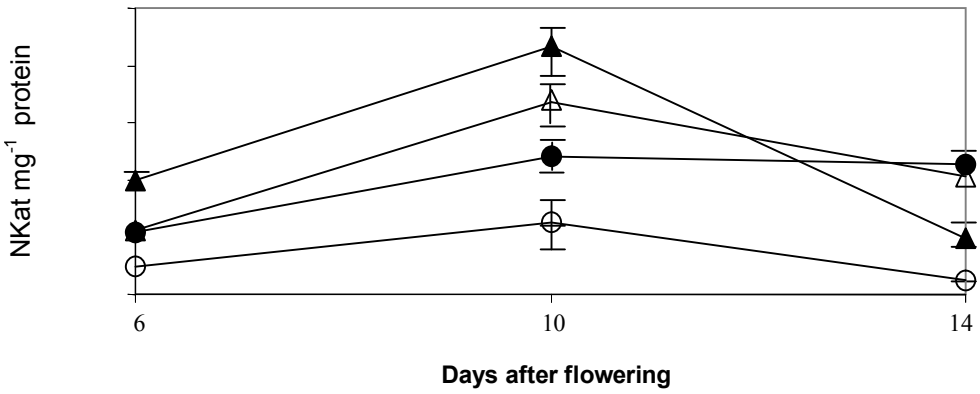

Figure 4: TLC plate of bracts and corollas extract from the different genotypes **1.** 1V8410; **2.** FRTS; **3.** 30154; **4.** 30302.

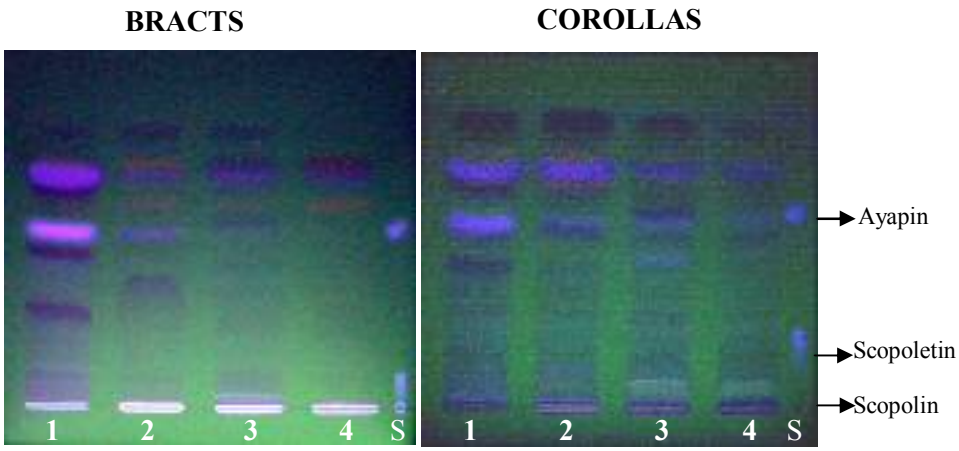

Figure 4: Phenolic content in healthy leaves (L) and bracts (B) of sunflower. Data correspond to the two lines tested: 20303 (■), and 1V8410 (□) at different stages. Values, expressed as µg equivalent of chlorogenic acid g^{-1} fresh weight, are mean of 8 replicates ± s.e.

5

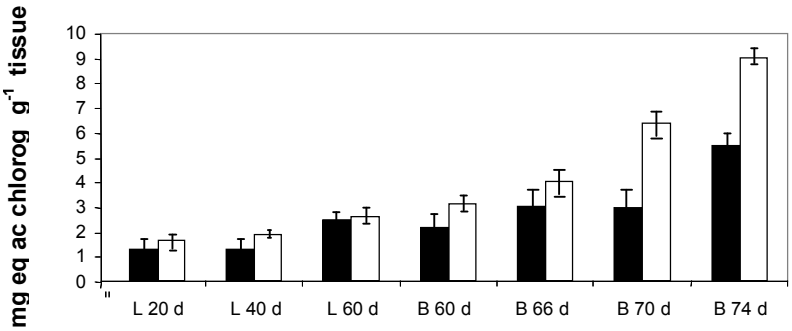

Figure 5: Hyphae growth in presence of bracts (A) and corollas (B) phenolic extracts. Values, expressed as length units (1 length unit = 10 μ) , are mean of 5 replicates (scoring 25 germinated spores per replication) ± s.e.

(1 length unit = 10 μ).