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Impact of polyphenolic extracts on resistance to fungal contamination in dry bean grains

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From their significant roles against the biotic and abiotic stresses, polyphenols have aroused a growing interest for a possible application in food industry. The aim of this study was the evaluation of antifungal activity of polyphenols extracted from two varieties of dry bean, presenting white (*Tima*) and red (*MGT djedida*) color. Determination of moisture for the two varieties showed that these grains are favorable for moulds growth. Purification and microscopic study of the isolated strains from beans allowed the identification of six moulds genera: *Alternaria*, *Aspergillus*, *Moniliella*, *Fusarium*, *Penicillium* and *Rhizopus.* Extraction of total polyphenols was carried by a polar solvent and their quantification was based on the reaction of Folin Ciocalteu. The total polyphenolic content was 0.40 ± 0.005 mg EAG/g for the red variety and 0.27 ± 0.005 mg EAG/g for the white variety. The antifungal activity carried by the methods of direct contact, dilution, and antifungal index (IA_{100}) demonstrated the antifungal capacity of polyphenols; most sensitive strains isolated are *Alternaria* sp., *Moniliella* sp. and *Rhizopus* sp.. The most active polyphenols are polyphenols extracted from presumably healthy red grains. Our results lend support of the creation of varieties bean high in polyphenols, which act as natural preservatives and bio-effective agents, and offer an alternative to chemical agents for protection of harvested beans in storage structures.

Key words: Polyphenols, antifungal activity, dry bean.

INTRODUCTION

Dry vegetables constitute with cereals is the backbone of the food system in the emergent countries. Unfortunately, considerable losses occur from harvest to storage. Contamination of moulds is the principal damage involving a quantitative and qualitative reduction of the food value which resulted to a fall in harvest yield and serious medical problems (Bulter and Day, 1998). In relation to this contamination, chemicals fungicides were employed to prevent the fungi growth (Phattayakorn and Wanchaitanawong, 2009). However, WHO prohibited the use of chemical fungicides, because of their undesirable toxicological effects in the long run, including cancerogenicity (Chahardehi et al., 2010). In the same way, consumer knowledge about the risks of these compounds on food, leads to seeking other conservation strategies which are valuable, natural and safe for health. Some research tasks showed that the capacity of a vegetable species to resist the attack of insects and micro-organisms is often correlated with the content of phenol compounds (Esekhiagbe et al., 2009). Phenolic compounds or polyphenols are one of the most important groups of compounds occurring in plants, where they are widely distributed, comprising at least 8000 different known structures. Polyphenols are also products of the secondary metabolism of plants (Esekhiagbe et al., 2009). These compounds are reported to exhibit antifun-

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gal activity. This hypothesis encouraged us to undertake this study seeking the correlation between the grain total polyphenolic content of two varieties of dry bean differentiated by their color and mould contamination.

MATERIALS AND METHODS

Vegetable material

This study was related to two varieties of dry bean. The first variety "MGT Djedida" is a neighboring variety "Algerian variety" characterized by dark red color with average size and consistent texture. The second variety "Tima" is an imported variety "French variety", characterized by white color and small size. For each variety of dry beans, a preliminary separation in two batches was carried out. A batch of the presumably healthy grains (HG) and another one for presumably contaminated grains (CG). This separation was carried out following a certain number of morphological criteria (color of tegument, aspect of grain, wrinkled grain, shape of grain, presence of scars or wounds on the grain) (Botton et al., 1990). To avoid the imprecise results, all the tests were repeated three times.

Water content

Water content of 5 g crushed grains was determined using a drying oven at 110°C until a constant weight was gotten and it is expressed as a percentage (Francois, 2004).

Isolation and identification of moulds genera

The mould isolation was carried by Ulster method or direct method, with this method, the moulds development is stimulated by incubating eight grains of each batch (HG and CG of the two varieties) directly deposited on a culture medium potato dextrose agar (PDA) at 30°C during three to four days in order to maintain the moulds growth (Botton et al., 1990). Genera identification was based on farming and morphological characters (Bouchet, 2005). Thus, the farming characters studied using binoculars were: speed growth, color of colonies, texture of thallus, presence or absence of exudations, color and color change of medium (Smith, 2002). *In situ* inspection of chains morphology of spores and mycelium was done in liquid medium between blade and plate (Botton et al., 1990).

Extraction and quantification of total polyphenols

Extraction of total polyphenols was carried out according to the protocol suggested by Luthria and Pastor-Corrales (2006) modified by Mujica et al. (2009). A sample (1 g) was suspended in 25 ml methanol- water 80:20 v/v acidified with 0.1% HCl. The mixture was left for 2 h at room temperature. Later, the mixture was centrifuged at 1800 *g* for 15 min, the methanol was decanted and the residue was re-extracted with 25 ml of fresh methanol. It was centrifuged again and the extracts were combined. The dry extract was recovered after a dry evaporation. The quantification of the total phenolic compounds was based on the Folin-Ciocalteu reaction, according to the method of Singleton et al. (1999). Folin-Ciocalteau reagent was diluted with distilled water (50% v/v). Dried bean extract (0.020 g) was re-dissolved in 1 ml methanol and 100 µl of this bean extract solution was mixed with 250 µl diluted Folin-Ciocalteau reagent. The reaction solution was left at room temperature for 5 min. Then, 250 µl of sodium bicarbonate solution (20 g/l) was added and the whole was brought to 2 ml with distilled water. The mixture was incubated at room temperature for 60 min. The absorbance of the solution was determined at 760 nm. The blank was prepared for each sample, by replacing our extracts polyphenolic by 80% methanol.

Quantification of total phenolics was based on a gallic acid standard curve generated by preparing (0.03 to 0.50 mg/ml) of gallic acid in methanol. Total phenolics were expressed as mg gallic acid equivalent (GAE) per gram of bean seed using the following formula (Madi, 2010):

Gallic acid equivalent (mg g^{-1} GAE) = concentration in gallic acid equivalent (mg/ml)* volume of extract (ml)/ mass of dried bean extract (g) (Madi, 2010).

Antifungal activity of the extracts polyphenolic

Antifungal activity was tested by direct contact method *in vitro* on six isolated strains. Inhibition rates (Hussin et al., 2009) and antifungal index 100 (AI_{100}) (Chang et al., 2008) were estimated on PDA medium with concentrations 30, 15, 7.5, 3.75 and 1.87 mg/ml. Inhibitory minimal concentration (IMC), fungicide concentration (FC) and fungistatic concentration (FSC) were given on PDB liquid medium (Derwich et al., 2010).

Inhibition rates

The inhibition rates were calculated as follows:

$PI (\%) = (A - B)/ A \times 100.$

Where, PI (%) is the inhibition rate expressed as a percentage; A is the diameter of growth zone in the test plate; B, the diameter of growth zone in the control plate.

Each experiment was performed three times, and the data were average (Bajpai et al., 2010). The polyphenolic extract is known as 'very active' when it has an inhibition ranging between 75 and 100%: the strain is known as very sensitive; active when it has an inhibition ranging between 50 and 74%: the strain is known as sensitive; moderately active when it has an inhibition ranging between 25 and 49%: the strain is known as limited; not active when it has an inhibition ranging between 0 and 24%: the strain is known as resistant (Alcamo, 1984).

Antifungal index 100 (AI_{100})

The concentration that inhibited 100% of the mycelium of fungi growth is expressed by the AI_{100}. The values of the AI_{100} were calculated graphically, where, the X-coordinate is represented by the extract polyphenolic concentration and the ordinate by the percentage of inhibition of moulds growth (Chang et al., 2008).

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by dilution method. Test samples of extracts polyphenolics were dissolved in DMSO. These solutions were serially diluted and were added to potato dextrose broth (PDB) to final concentrations of 15 to 7.5 to 3.75 to 1.875 mg/ml. A 10 µl spore suspension of each test strains was inoculated in the test tubes in PDB medium and incubated for two to seven days at 30°C. The control tubes containing PDB medium were inoculated only with fungal spore suspension. The minimum concentrations at which no visible growth was observed were defined as the MICs, which were expressed in mg/ml (Bajpai

et al., 2008).

Fungicide concentration (FC) and fungistatic concentration (FSC)

Petri dishes containing 20 ml of PDA were inoculated with 10 µl of each tube presenting a total inhibition of the fungi growth. The follow-up of the growth is carried during 1 to 4 days at 30°C. When there is no continuation of growth; the concentrations are known as fungicides (CFs) (Zarrin et al., 2010) and the concentrations for which there is growth are known as fungstatics (CFSs) (Bajpai et al., 2010).

Statistical analysis

Obtained results were subjected to an analysis of variance and the test of correlations (with the threshold of significance 0.05), using software XLSTAT (2008).

RESULTS AND DISCUSSION

Water content

Revealed water content varied from 11.47 to 12.50% and it is different from that noted by Laîb (2009) (higher than 15%). Indeed, this parameter depends on several factors such as variety, harvest period and storage temperature. Referring to Multon (1982), moisture percentage required during grain storage should not exceed 11% to avoid fungi deterioration. According to the obtained results, the analyzed dry bean samples contained sufficient water and are favorable to the development of moulds. On the varietal level, variance analysis indicates a significant difference ($p \leq 0.05$) between the two varieties. On the sampling plan of the same variety, no significant difference was noticed between healthy sample and contaminated sample for white variety; on the other hand it was significant for red variety (Table 1).

Isolation and identification of moulds

43 strains were revealed from the analyzed samples and they were gathered in six genera: *Alternaria*, *Aspergillus*, *Fusarium*, *Moniliella*, *Penicillium* and *Rhizopus* (Figure 1). Four of these genera were already identified and quoted by Laîb (2009) in dry beans exposed and commercialized in the region of Skikda (city in northeastern Algeria). This could be due probably to inappropriate conditions of exposure of these grains in the market, generally without adequate packing; this situation is practically the same in the Algerian East. Other studies on leguminous fungi flora showed that the moulds storage of dry beans in India included the genera: *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Penicillium, Rhizoctonia, Stemphylium* and *Trichoderma*. The species belonging to the genera *Penicillium* and *Eurotium* were common for bean from Taiwan, but in Canadian beans, the widespread mycetes were *Alternaria*, *Fusarium* and *Rhizoctonia* (Tseng et al., 1995). Grains of red variety appeared contaminated with 46.51% (contaminated by 20 strains among the 43 isolated strains, namely the genera *Alternaria*, *Penicillium* and *Rhizopus*) and those of white variety with 53.49% (contaminated by 23 strains among the 43 isolated strains, namely the genera *Aspergillus*, *Fusarium*, *Moniliella*, *Penicillium* and *Rhizopus*). Even if the two varieties in common do not have the same conditions of harvest and storage, the red variety seems to be more resistant to the fungi contamination.

Considering chemical composition, the white variety offers the advantage of containing a higher rate of proteins and glucides than the red variety (Kassemi, 2006). Several authors in particular (AbdelMassih, 2007) showed a positive correlation between fungi contamination and richness in the grains in its two elements. In the same way, the red variety contains more phenolic compounds, which explain their color (Beninger and Hosfield, 2003), and are known by their resistant effect to the pathogenic attacks (Cherif et al., 2007). The five genera identified on the analyzed samples (*Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus*) have strains toxinogenes. The harmful effects of their mycotoxins is very wide; mutagen, necrosing, neurotoxic, heaptotoxic and hematotoxic (Brochard and Bâcle, 2009).

Evaluation of total polyphenolic content

Total contents of phenolic compounds values are recapitulated in Table 2. Concerning the red variety, the analysis of the variance does not show any significant difference ($p \leq 0.05$) between healthy sample and contaminated sample. At the reverse, the white variety presented a significant difference ($p \leq 0.05$) between healthy sample and contaminated sample. As for the varietal difference, the same observation was made by Laparra et al. (2008), YingTan et al. (2008) and Xu and Chang (2009); colored dry beans were found richer in polyphenols than white dry beans. The color of the bean grain is determined by the presence and the concentration of the flavonol glycosides, the anthocyanin and the condensed tanins (Reynoso et al., 2006). The red dry bean genotype has higher contents in anthocyanin than the white genotype. This difference is due probably to the anthocyanin, which contributes significantly to the coloring of grains (Horbowicz et al., 2008).

However the variety is that we notice clearly that healthy samples are richer in total polyphenols than contaminated samples. It seems that the polyphenols are present more in significant quantity plus the less contamination. This indicates the existence of a positive correlation between contamination rate and phenol total content in dry bean grains. This correlation was reported by

Table 1. Difference analyze by Tukey test between dry bean samples for water content ($p \leq 0.05$).

Sample	Estimated average		
	Healthy sample	Contaminated sample	Variety average
Red variety	11.47±0.15[c]	12.50±0.05[a]	11.98±0.10
White variety	11.60±0.05[b]	12.00±0.30[b]	11.80±0.17

Same letter means absence of significant difference.

Table 2. Polyphenolic total content of the extracts (mg GAE/g).

Sample	Quantity of total phenol (mg GAE/g)	Average (mg GAE/g)
Red healthy bean	0.42±0.007[a]	0.40±0.005
Red contaminated bean	0.39±0.003[a]	
White healthy bean	0.32±0.008[b]	0.27±0.005
White contamined bean	0.22±0.002[C]	

The same letter means absence of significant difference.

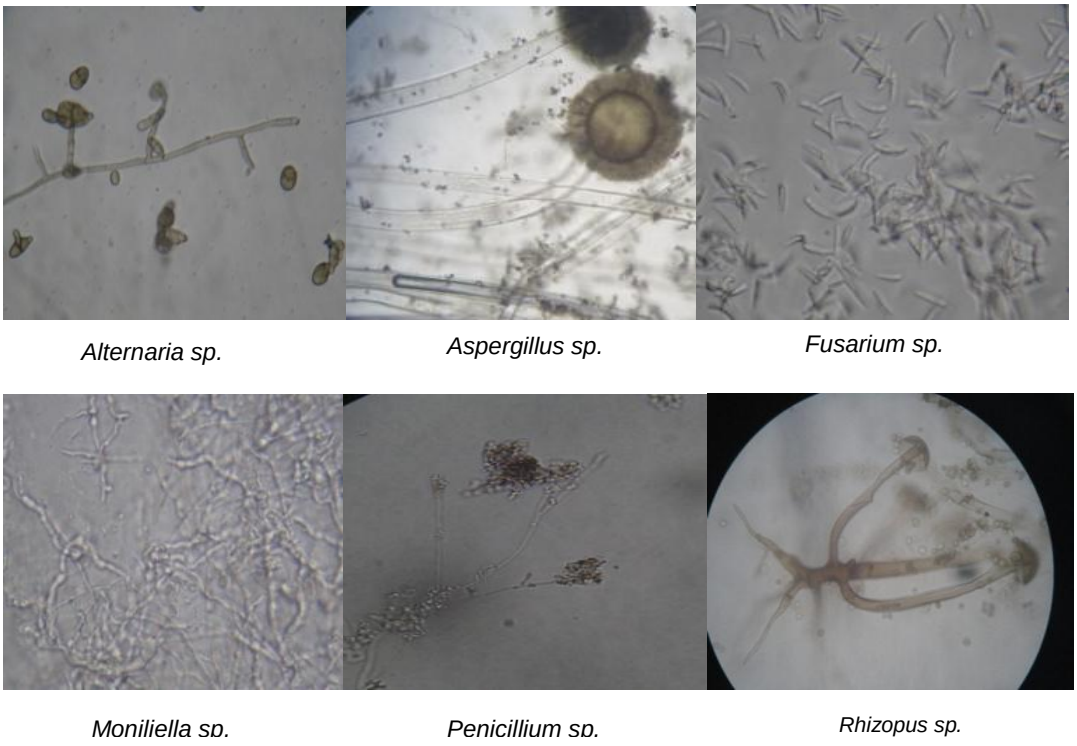

Figure 1. Photographs of some mould strains isolated from the two varieties of dry beans (x40).

several plant bioactive substance researches; for example, Abad et al. (2007) allots this property to flavonoides, like flavonol glycosides, a major class of polyphenolic compounds of dry bean grains.

Several factors can influence the polyphenolic contents. There are climatic and environmental factors (Ebrahimi et al., 2008), genetic factors, experimental factors (Miliauskas et al., 2004) and because of the differences in the methods of extraction and determination and in the ways of expressing results between various authors (Lee et al., 2003), it is difficult to compare our data with those from literature. For example, Cardador et al. (2002) found the total phenolic content to be 2.09 mg of catechin equivalents per gram of seeds. The same

Table 3. Stamp correlations [*Pearson (N)*] between the principal analyses carried out on the dry bean samples (with a threshold of significance of 0.05).

Variable	Water content	Rate of contamination	Polyphenols content
Water content	**1**	0.773*	-0.085
Rate of contamination		**1**	-0.698*
Polyphenols content			**1**

*Significant correlation.

set of authors in another publication have reported the concentrations of phenolic compounds in six bean cultivars in the range of 3.28 to 16.61 mg of catechin equivalents per gram of seeds (Oomah et al., 2005).

Correlations between rate of contamination, moisture and the polyphenolic content

Correlation matrix (Table 3) indicates a correlation positively significant (r = 0.773) between the water content and the rate of contamination of the grains. This correlation can be explained by the fact that the majority of moulds prefer raised water contents, in particular, in the germination phase which requires a more significant contribution of water compared to the growth phase (Basset, 2009). On the other hand, the rate of contamination is correlated negatively and in a significant way (r = -0.698) with polyphenolic content. This opposite correlation can be interpreted in two manners; either the high percentage of contamination involves a reduction of the total polyphenolics content, or it is the presence of a significant quantity of total polyphenols which causes a reduction in the contamination rate. While referring to the bibliography, this correlation is due probably to fungicide and fungistatic effects of polyphenols (Kawamura et al., 2010).

Antifungal capacity test

Inhibition rate

Inhibition rates obtained are represented in Figure 2. Through, the various percentages of inhibition obtained in the study, polyphenols showed variable activities on the tested strains. Concerning *Alternarias* sp*.,* the polyphenols extracted from contaminated red bean (PHRC) and from contaminated white bean (PHBC) gave inhibition rates higher than those of polyphenols of healthy red bean (PHRS) and of healthy white bean (PHBS). The latter were higher than 50%. This is probably explained by the absence of contamination by this strain in the case of contaminated white bean and reduction of contamination rate for contaminated red bean if one compares the contamination rate with that of healthy red bean. As for *Fusarium* sp*.*, it appeared to be not very sensitive to polyphenols extracted from contaminated white bean for the five concentrations. The other types of polyphenolic extracts are moderately active and exert a depigmentation of the air mycelium of the strain considered. For *Moniliella* sp*.,* three types of polyphenolic extracts are revealed to be very active; polyphenols extracted from healthy red bean, contaminated red bean and polyphenols extracted from contaminated white bean for concentrations 1.87 to 15 mg/ml (inhibition rate > 75%) except for the PHRC which was active for the concentrations 0.94 and 1.87 mg/ml.

The healthy white bean extracts polyphenolic (PHBS) are revealed to be active (inhibition rate > 60%) for the five concentrations. This justifies the absence of this kind in our samples except for the weak even negligible rate of contamination for the healthy white bean (seldom isolated kind). For a concentration of 15 mg/ml, the polyphenolic extracts of the healthy samples of the two varieties of dried bean show rates of inhibition higher than 50% on the genera *Rhizopus.* This last result was characterized by a reduction in the sporulation. For the other concentrations, this kind appeared limited and the polyphenolic extracts are known as fairly active. Strains belonging to *Penicillium* sp*.* and *Aspergillus* sp*.* have an arbitrary growth that prevented us from calculating their inhibition rates because of the dispersion of the spores; consequently, we could not measure their diameters.

Determination of antifungal index (A I_{100})

Antifungal index (AI100) of the polyphenolic extracts is given in Table 4.

Inhibitory minimal concentration (IMC)

Inhibitory minimal concentration (IMC) is calculated in order to define the antifungal effectiveness of the polyphenolic extracts (Tiwari et al., 2009). The esults are shown in Table 5. Studied strains do not have the same sensitivity to polyphenolic extracts of the whole dry bean grains. Strains belonging to genera *Alternaria*, *Moniliella* and *Rhizopus* are most sensitive to polyphenolic extracts; in particular, those of the healthy red variety.

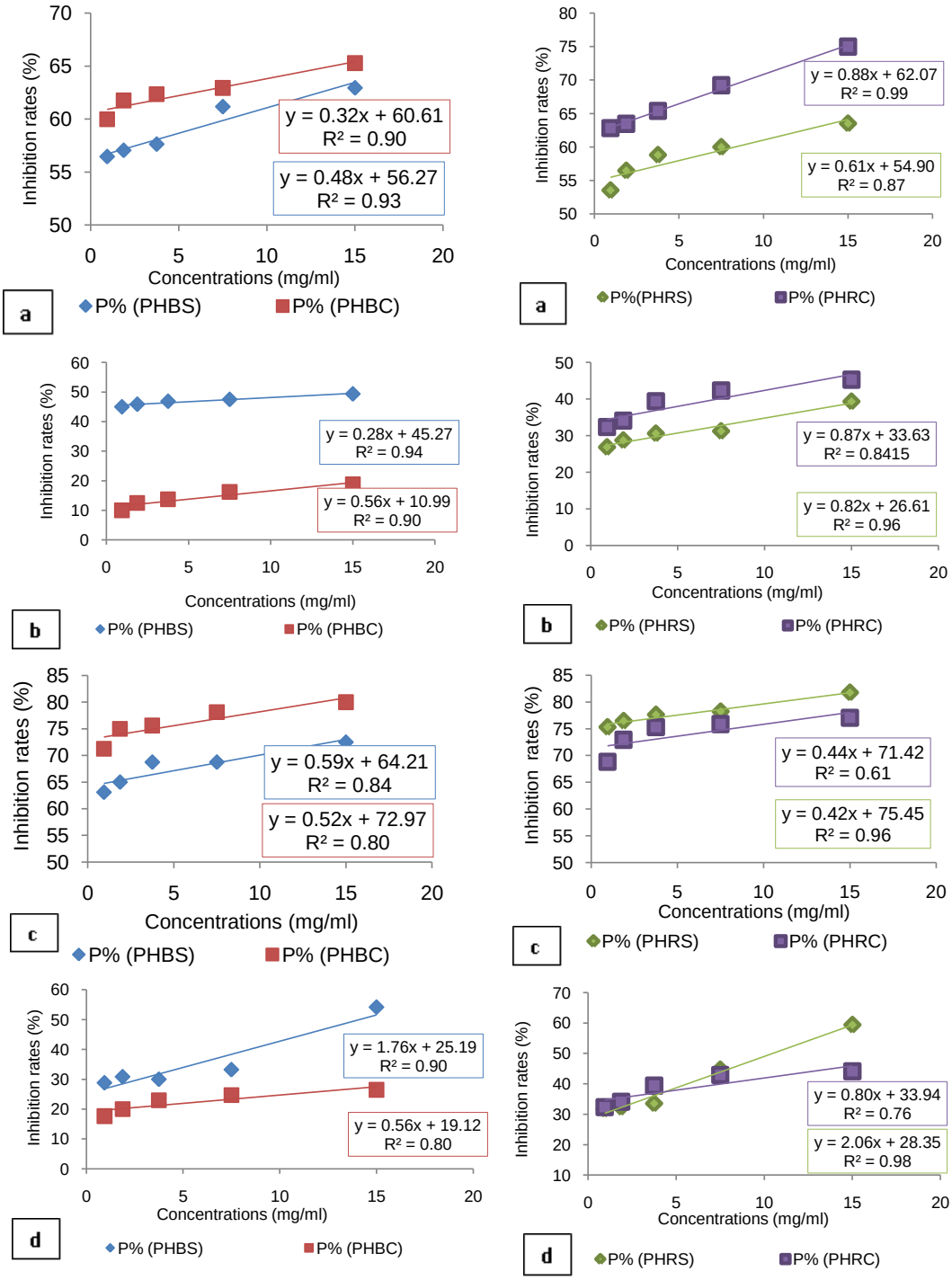

Figure 2. Inhibition rates of polyphenolic extracts against tested strains. a. *Alternaria* sp.; b. *Fusarium* sp.; c. *Moniliella* sp.; d. *Rhizopus* sp. PHBS, Polyphenolic extracts from white healthy bean; PHBC, polyphenolic extracts from white contamined bean; PHRS, polyphenolic extracts from red healthy bean; PHRC, polyphenolic extracts from red contamined bean.

Fungistatic and fungicide concentrations

Values of fungicide and fungistatic concentrations (Table 6) confirm the results obtained in solid medium by the direct contact method. This antifungal activity seems to be related to the presence of certain chemical functions, like the presence of an aromatic nucleus and an hydroxyl group in the structure of the phenolic compounds

Table 4. Antifungal index (AI_{100}) of the polyphenolic extracts.

Strain	IA_{100} (mg/ml)			
	PHRS	PHRC	PHBS	PHBC
Alternaria sp.	73.93	43.10	91.10	123.09
Aspergillus sp.	ND	ND	ND	ND
Fusarium sp	89.5	76.29	195.46	158.95
Moniliella sp	58.45	64.95	60.66	51.98
Penicillium sp.	ND	ND	ND	ND
Rhizopus sp.	34.78	82.57	42.50	144.43

ND, Not determined; PHBS, polyphenolic extracts from white healthy bean; PHBC, polyphenolic extracts from white contamined bean; PHRS, polyphénolic extracts from red healthy bean; PHRC, polyphenolic extracts from red contamined bean.

Table 5. Inhibitory minimal concentration (mg/ml) of polyphenolic extracts in liquid medium.

Strain	IMC (mg/ml)			
	PHRS	PHRC	PHBS	PHBC
Alternaria sp.	30	30	15	30
Aspergillus sp.	30	30	30	30
Fusarium sp.	30	30	30	30
Monilleila sp.	1.87	7.50	15	15
Penicillium sp.	15	30	15	30
Rhizopus sp.	15	30	15	30

PHBS, Polyphenolic extracts from white healthy bean; PHBC, polyphenolic extracts from white contamined bean; PHRS, polyphenolic extracts from red healthy bean; PHRC, polyphenolic extracts from red contamined bean.

Table 6. Fungistatic concentration (FSC) and fungicide concentration (FC) in mg/ml.

Strain	FC	FSC	FC	FSC	FC	FSC	FC	FSC
	PHRS		PHRC		PHBS		PHBC	
Alternaria sp.	30	ND	ND	30	30	15	30	ND
Aspergillus sp.	30	ND	30	ND	30	ND	30	ND
Fusarium sp.	30	ND	30	ND	30	ND	30	ND
Moniliella sp.	3.75	1.87	7.5	ND	15	ND	15	ND
Penicillium sp.	ND	15	ND	15	30	15	ND	30
Rhizopus sp.	15	ND	30	ND	30	15	ND	30

ND, Not determined; PHBS, polyphenolic extracts from white healthy bean; PHBC, polyphenolic extracts from white contamined bean; PHRS, polyphenolic extracts from red healthy bean; PHRC, polyphenolic extracts from red contamined bean.

(Shirazd et al., 2011).

Conclusion

By their chemical composition, dry beans undoubtedly appear among the raw materials mostly exposed to fungi contamination. Polyphenols are among the grain components which is able to play a role in its defense against biotic and abiotic stress. Antifungal test carried out, *in vitro*, shows that the polyphenolic extracts of dry beans tested have antifungal, fungicide and fungistatic effects variable in function of the strain and the amount applied. The most significant mould was *Alternaria* and the most active polyphenols were those extracted from healthy red bean. Creation of varieties with grains rich in polyphenols can improve resistance to the fungi infestation.

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