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Proceeding Paper

Influence of Cultivation Areas on the Seed-Borne Pathogens on Two Local Common Bean Ecotypes of "Fagioli di Sarconi" PGI (*Phaseolus vulgaris* L.) †

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Abstract: The "Fagioli di Sarconi" common beans, typical of Basilicata Region (Southern Italy), include different ecotypes protected by the European Union with the mark PGI (Protected Geographical Indication). The study aimed to determine the presence of seed-borne pathogens, isolated from two ecotypes of "Fagioli di Sarconi" common beans, "Ciuoto" and "Cannellino rosso", in two different cultivation areas during the years 2018 and 2019, for identifying genotypes resistant and well adapted to climatic changes. The seeds were evaluated for seed-borne pathogens screening by using three validated seed health testing methods, according to the 2020 International Rules for seed testing. The washing test identified 18 fungal pathogens, different for ecotype and year of observation; the 1% sodium hypochlorite treatment strongly reduced the contaminants. With the blotter test, several saprophyte pathogens were found. Between paper test, specific for detecting the *C. lindemuthianum*, revealed the presence of this pathogen for both ecotypes and years, in all areas, and individuated some bacteria, too. In conclusion, this work highlighted differences by the two PGI common bean ecotypes in response to seed-borne pathogens resistance and environmental change due probably to their different thickness and polyphenolic content of integument.

Keywords: common bean; seed-borne pathogens; climatic changes; genotypes resistance

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1. Introduction

Phaseolus vulgaris L., known as the common bean, is the second most important legume in the world for food purposes thanks to its high nutritional value in terms of content of protein, vitamins, zinc, iron, and fiber [1,2]. It is widespread over a large area between 52° N and 32° S of latitude, at low altitudes until sea level (USA and Europe) and high altitude (South America) [3]. In Italy, the cultivated area is ~24,000 ha (FAOSTAT 2018. Statistical database 2018, Available at http://faostat.fao.org, accessed on 22 April 2020). The Basilicata Region (South Italy) is invested with 200 ha in the upper Agri Valley and Mercure Valley (Sarconi). "Fagioli di Sarconi" common beans includes about 21 different ecotypes protected by the European Union with the mark PGI (Protected Geographical Indication) [4]. Seed-borne pathogenic fungi and bacteria can inhibit germination, kill seedlings, or reduce plant growth by damaging the roots and vascular system, and affecting the transport of water and nutrients [5–7]. The main seed-borne pathogenic fungi that cause losses of yield and quality of common bean in South Italy are *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cav, *Rhizoctonia solani* (Cooke) Wint, *Fusarium oxysporum* (Schlecht.) Snyd

& Hans. and *F. solani* (Mart.) Snyd. & Hans. This study aimed to determine the presence of seed-borne pathogens through different diagnostic methods, isolated from seeds of two ecotypes of "Fagioli di Sarconi" common beans, "Ciuoto" and "Cannellino rosso", collected from two different cultivation areas of Basilicata Region during the years 2018 and 2019, for identifying genotypes resistant or little susceptible to seed-borne pathogens and well adapted to climatic changes.

2. Experiments

Sample collection. During the years 2018 and 2019 the phytosanitary status of seeds of two ecotypes of "Fagioli di Sarconi" PGI, "Ciuoto" and "Cannellino rosso", characterized by a determinate habitus, was evaluated. The samples were collected from two different areas located in the upper Agri Valley: Sarconi and Paterno (Potenza, Italy). The meteorological data (rain, temperatures, and relative humidity) for the Agri Valley territory, provided by the agrometeorological station of the Agenzia Lucana per lo Sviluppo e l'Innovazione in Agricoltura (ALSIA) of the Basilicata Region, were considered. Seeds were stored at room temperature (25° ± 2 °C) until use.

Isolation of seed microflora. The phytopathological analysis of seeds was carried out with three different methods, according to the 2020 International Rules for seed testing [8].

Washing test. The washing test method allows identification of the spores of the fungi contaminating the seed surface [8,9]. For each test, 400 seeds (4 replicates × 100) were considered. The separation of spores and mycelium from the integument surface was obtained by stirring the seeds in presence of sterile, distilled water. After filtration, the suspension was centrifugated at 5000× *g* for 11 min. The precipitated was resuspended in 200 µL of sterile, distilled water, and samples were set up for observation under the optical microscope. In addition to this procedure, seeds were sown on plates of 90 mm in diameter containing Potato Dextrose Agar (PDA) and the antibiotics ampicillin and stremptomycin (Sigma-Aldrich, Milan, Italy), and incubated at 22 °C in the dark, for 5–8 days. Thereafter, slides were prepared for observation under the optical microscope.

Blotter test. Discs of absorbent filter paper of the same diameter were inserted in Petri dishes with a diameter of 140 mm and moistened with 2 mL of sterile, distilled water. Four-hundred seeds (4 replicates × 100) were disinfected with a 1% sodium hypochlorite solution for 10 min and distributed on the moistened paper. The plates were incubated at 20 °C for 10 days with alternating 12 h of NUV light (near-UV light) and 12 h of dark. After the incubation, the seeds were examined under the optical microscope. Untreated seeds represented the control.

Between paper test. For the identification and subsequent characterization of *Colletotrichum* sp. species, the indicated protocol was used [8] with small modifications. Two-hundred seeds (4 replicates × 50) were immersed for 10 min in a 1% sodium hypoclorite solution and then air-dried. Subsequently, 4 subsamples of 50 seeds were placed between two double sheets of paper toweling and soaked in sterile distilled water. The paper toweling was folded lengthwise and covered with a polyethylene sheet to keep a high moisture content during incubation (7 days at 20 °C). Then, the seeds and cotyledons were observed, and black depressed areas with well-defined contours searched. The typical signs of the anthracnose pathogen presence were checked (acervules with or without bristles).

Identification of pathogens. After the incubations and the slides preparation, the macroscopic (appearance of the colony and mycelium) and microscopic characteristics (appearance of the mycelium, presence or absence of septa in hyphae, color, shape and size of conidia, reproductive structures) were revealed under the optical microscope (Axioskop, Zeiss, Germany) with objectives 20×, 40×, and 100×. For the microscopic and morphological identification of the fungal isolates, scientific literature and mycological atlases with related taxonomic keys were used [10–18].

Statistical analysis. All data, including climatic parameters (rain, minimum air temperature, maximum air temperature, average air temperature, minimum relative humidity, maximum relative humidity, average relative humidity, and potential evapotranspiration)

for the two years of observation 2018 and 2019, were tested with Stat Plus v.7 (analystsoft, Statplus: mac-statistical analysis programs) [19] and GenAlex v.6.5 [20] for analysis of variance, ANOVA.

3. Results

3.1. Meteorological Parameters

The rain, temperature, and relative humidity data for the Agri Valley territory during the cultivation period, from 1 June to 30 November for both years 2018 and 2019, are summarized in Table 1.

Table 1. Meteorological parameters provided by the agrometeorological station of ALSIA in Agri Valley for 2018 and 2019 years and their one-way ANOVA Test.

Meteorological Parameter	2018				2019				Pr (>F)
	Min	Max	Range	Mean	Min	Max	Range	Mean	
Rain (mm)	0.00	38.60	38.60	2.09	0.00	56.40	56.40	1.69	0.530
Temperature air (°C)									
Minimum	−4.30	19.80	24.10	10.03	−0.50	16.50	17.00	9.58	0.301
Maximum	8.40	36.40	28.00	26.13	11.00	39.40	28.40	27.78	0.016 *
Average	2.83	25.78	22.94	17.20	6.28	25.93	19.64	18.08	0.094
Relative humidity (%)									
Minimum	19.00	90.00	71.00	42.39	13.00	91.00	78.00	37.58	0.001 ***
Maximum	67.80	100.00	32.20	96.96	82.00	99.80	17.80	96.96	0.998
Average	46.25	96.42	50.17	75.09	49.67	96.79	47.13	71.25	0.000 ***
Evapotranspiration (mm)	0.76	8.13	7.37	4.44	0.90	8.08	7.18	4.87	0.049 *

*** Pr < 0.001; * Pr < 0.05.

3.2. Isolation and Identification of Seed Mycoflora with Washing Test

We found that there was 18 seed-borne fungal species contaminating or infecting the two ecotypes of "Fagioli di Sarconi" common bean PGI seeds in the two lucanian farms, located in the two different area and during the two years 2018 and 2019, as reported in Table 2.

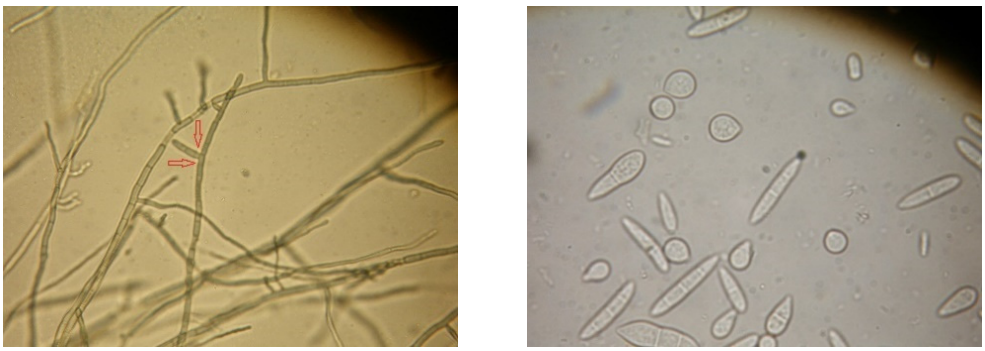
Table 2. Seed-borne fungal species detected (+) or not (−) as isolated by washing test on the two ecotypes of "Fagioli di Sarconi" common bean PGI seeds.

Year	2018				2019			
Ecotype	Ciuoto		Cannellino Rosso		Ciuoto		Cannellino Rosso	
Locality	Sarconi	Paterno	Sarconi	Paterno	Sarconi	Paterno	Sarconi	Paterno
Fungal microflora								
Alternaria sp.	+	−	−	−	−	+	+	−
Alternaria alternata	−	−	−	−	−	+	−	+
Aspergillus sp.	−	−	+	+	−	−	−	−
Aspergillus flavus	−	−	+	+	+	−	−	−
Aspergillus niger	+	−	−	−	−	−	+	−
Cladosporium cladosporioides	−	−	−	+	+	+	+	−
Botritys sp.	−	−	+	−	−	+	−	+
Colletotrichum lindemuthianum	−	+	−	−	−	+	−	−

Table 2. *Cont.*

Year	2018				2019			
Fusarium oxysporum	–	–	–	–	–	+	–	+
Fusarium solani	–	+	–	–	–	+	–	–
Mucor hiemalis	+	–	–	–	–	–	+	–
Penicillium sp.	–	–	+	+	–	+	+	+
Penicillium expansum	–	+	–	–	–	+	–	–
Rhizoctonia solani	–	+	–	+	+	+	+	+
Rhizophus nigricans	+	–	–	–	–	–	–	–
Trichoderma harzianum	-	–	–	+	–	–	–	–
Tricoderma viridae	+	–	–	–	–	–	–	–
Uromyces appendiculatus	–	–	–	–	–	+	–	+

Microscopic morphological structures identifying of the fungi *Rhizoctonia solani* isolated from "Cannellino rosso" ecotype, and *Fusarium oxysporum* isolated from"Ciuoto", located in Paterno in 2018 and 2019, respectively, are shown in Figure 1.



(**a**) (**b**)

Figure 1. (**a**) Secondary conidiophore branches, on the main hypha of *Rhizoctonia solani* (resolution 40×), arranged at right angle and restricted in their insertion point (red arrows). (**b**) Macroconidia and microconidia of *Fusarium oxysporum* (resolution 40×).

3.3. Identification and Incidence of Rhizoctonia solani on Treated and Untreated Seeds with Blotter Test, and of Colletotrichum lindemuthianum, Fusarium oxysporum and Bacterial Disease with between Paper Test

In Table 3, the seed-borne pathogenic fungi and bacteria considered the most dangerous in determining losses of yield and quality in the common bean are reported. For this reason, their disease incidence was also considered. In fact, the percentage of rhizoctoniosis from *Rhizoctonia solani*, or of symptoms of *Colletotrichum lindemuthianum*, *Fusarium oxysporum*, and bacterial disease on integument and cotyledons were depicted.

Table 3. Seed-borne fungal species detected (+) or not (−) as isolated by blotter and between paper tests on the two ecotypes of "Fagioli di Sarconi" common bean PGI seeds. The percentage of disease incidence indicates rhizoctoniosis from *Rhizoctonia solani* and fungal or bacterial symptoms for the other pathogens.

Year	**2018**				**2019**			
Ecotype	**Ciuoto**		**Cannellino Rosso**		**Ciuoto**		**Cannellino Rosso**	
Locality	**Sarconi**	**Paterno**	**Sarconi**	**Paterno**	**Sarconi**	**Paterno**	**Sarconi**	**Paterno**
BLOTTER METHOD								
Rhizoctonia solani								
treated seed	+	+	+	+	+	+	+	+
Disease incidence (%) [1]	27	23	8	15	12	15	4	8
untreated seed	+	+	+	+	+	+	+	+
Disease incidence (%) [1]	19	35	24	27	11	23	12	24
BETWEEN PAPER TEST								
Fusarium oxysporum	+	+	−	+	−	+	−	+
Disease incidence (%) [2]	1	4	0	2	0	10	0	6
C. lindemuthianum	+	+	+	+	+	+	+	+
Disease incidence (%) [2]	4	40	8	20	4	65	4	30
Bacterial disease								
P. syringae pv. *phaseolicola*	+	+	+	+	+	+	+	−
X. campestris pv. *phaseoli*	+	+	+	−	−	+	−	+
Disease incidence (%) [2]	28	30	18	24	12	12	6	16

[1] The differences of rizzottoniosis from *Rhizoctonia solani* between untreated and treated seeds are significant for $\chi^2 = 87.04^{***}$, at $p < 0.000$; [2] the differences of symptoms between ecotype, year and locality are significant for $\chi^2 = 9.71^{*}$, at $p \leq 0.046$ for *C. lindemuthianum*, for $\chi^2 = 8.50^{*}$, at $p < 0.037$ for *F. oxysporum*, and for $\chi^2 = 10.84^{*}$, at $p \leq 0.028$ for the bacterial disease.

The typical damping-off symptoms of *Rhizoctonia solani* on stem of "Cannellino rosso" common bean ecotype, located in Sarconi (2019), revealed by blotter test is shown in Figure 2.

Figure 2. Hydropic halo and felted mycelium, wrapped in a ring, by *Rhizoctonia solani* on the stem of a "Cannellino rosso" ecotype seedling.

In Figure 3, some microscopic morphological structures identifying of the fungus *Colletotrichum lindemuthianum* isolated from "Ciuoto" ecotype, located in Paterno (2018) and typical symptoms on seeds of anthracnose and fusariosis are reported, all detected by between paper test.

(**a**) (**b**) (**c**)

Figure 3. (**a**) Acervulus of *Colletotrichum lindemuthianum* (similar to sporodochium) in vitro, with bristles in evidence (resolution 20×); (**b**) Symptoms of anthracnose on teguments and cotyledons by *C. lindemuthianum* on "Ciuoto" ecotype located in Paterno (2018); (**c**) Mycelium of *Fusarium oxysporum* on tegument, with a characteristic vinous aura, on "Ciuoto" ecotype located in Paterno (2019).

In addition to the above-described fungi and bacteria, the blotter test was able to individuate, in the 1% sodium hypochlorite-treated seeds, the following seed-borne fungi: *Aspergillus niger* (2018, Paterno, both ecotypes; 2019, Sarconi, "Cannellino rosso"); *Fusarium solani* (2019, Paterno, both ecotypes); *Penicillium expansum* (2018, Paterno, "Ciuoto"). Instead, the following fungi were detected in untreated seeds: *Aspergillus flavus* (2019, Sarconi, "Cannellino rosso"); *Aspergillus niger* (2018, Paterno, both ecotypes; 2019, Sarconi, both ecotypes); *Cladosporium cladosporioides* (2019, Sarconi, "Cannellino rosso"); *Fusarium solani* (2018, Paterno, "Ciuoto"; 2019, Paterno, "Cannellino rosso"); *Mucor hiemalis* (2018, Sarconi, "Ciuoto" and Paterno, both ecotypes; 2019, Paterno, both ecotypes); *Penicillium* sp. (2018, Sarconi, "Cannellino rosso"); *Penicillium expansum* (2018, Paterno, "Ciuoto"); *Rhizophus nigricans* (2018, Sarconi, "Ciuoto").

4. Discussion

The health of the seed represents a fundamental point for obtaining good production. The presence of pathogens in or on the seed influences all the vegetative and productive phases. Seeds intended for food may not only have a little nutritional value but may be contaminated with mycotoxins. Therefore, the health of the seed is an indispensable requirement for the productivity and quality of the crop. For the isolation and identification of the seed-borne pathogens of common beans, diagnostic methods with different sensitivities were used. The test of washing proved to be the most effective and sensitive compared to the others. In fact, the washing test allowed to detect most of the fungi contaminating the tegument and infecting the cotyledons and the embryo. The seed-borne fungal agents were 18. Most of these (*R. solani, C. lindemuthianum, F. oxysporum, F. solani, U. phaseoli*) are of great importance due to their damages on the seed in pre-emergency and post-emergency, so causing economic losses [21]; not less dangerous are others fungal pathogens detected (*Alternaria alternata, Penicillium* sp., *Aspergillus flavus, A. niger, Mucor hiemalis, Fusarium* sp.), determining substantial problems linked to the mycotoxins production during the post-harvest storage [16]. This method also allowed to identify beneficial fungi (*Trichoderma harzianum* and *T. viridae*) known to be used as antagonist of harmful pathogens [22–24].

The blotter method, unlike the washing test, was more selective towards some pathogens with saprophytic behavior (*Aspergillus* sp., *Cladosporium* sp., *Botritys* sp., *Mucor* sp., *Rhizophus* sp.). The treatment of the seeds with 1% Na-hypochlorite allowed a reduction of most of fungal and bacterial microflora adhering to the seed but did not allow to eliminate the systemic pathogens infecting the internal structures of the seed, such as *C. lindemuthianum*, *F. solani*, *F. oxysporum*, and *R. solani*.

The between paper method, applied as international protocol specific for detecting *C. lindemuthianum*, revealed its presence on both ecotypes, years, and areas.

Regarding the thermo-hygrometric data collected during the entire crop cycle, the mean minimum relative humidity values for the years 2018 and 2019 were 42.39% and 37.58%, respectively, while the average relative humidity values were 75.09% and 71.25%. These values showed little, but significant difference between the two years. The average

temperature did not show significant difference between the two years, while were different the maximum temperatures. This finding could explain the prevalent presence of the hygrophilous fungal species detected during the year 2018, when their spores were able to germinate at humidity values of 55–75%, such as *Alternaria sp.*, *A. alternata*, *C. cladosporioides*, *Penicillium* sp., *Fusarium* sp., *Colletotrichum* sp., and *R. solani* [15,16].

Concerning the incidence of anthracnose by *C. lindemuthianum*, of fusariosis by *F. oxysporum*, and of bacteriosis, the results highlighted that the "Cannellino rosso" ecotype was less susceptible to plant diseases, compared to the "Ciuoto" one. The reasons are to be found not only in the higher integument thickness of "Cannellino rosso" respect to others local ecotypes [25,26], but probably also to the higher content of polyphenolic compounds (hydrolysable tannins and condensed tannins) present in the tegument, considered protective factors for the seed against seed-borne pathogens [27]. On the contrary, only the Na-hypochlorite treatment induced in the "Cannellino rosso" ecotype a less susceptibility to rhizoctoniosis by *R. solani*.

5. Conclusions

The phytosanitary screening of seeds of "Fagioli di Sarconi" PGI common bean ecotypes "Ciuoto" and "Cannellino rosso", cultivated in the areas of Paterno and Sarconi in Agri Valley, allowed to detected part of the fungal and bacterial microflora harmful to the quality of the seeds. It was possible to individuate the "Cannellino rosso" as less susceptible to the majority of diseases. Indeed, the incidence of the pathogens analyzed in the current work was area-, environment-, and ecotype-dependent. The present study represents a baseline information for further studies and management of seed-borne diseases associated with "Fagioli di Sarconi" PGI common bean.

Author Contributions: V.B. and M.N. conceived and designed the experiments; V.B. performed the experiments; V.B., A.V., G.L., T.G. and M.N. analyzed the data; S.M., G.L. and M.N. contributed reagents/materials/analysis tools; V.B., A.V. and M.N. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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