

# Influence of Cultivation Areas on the Seed-Borne Pathogens on Two Local Common Bean Ecotypes of “Fagioli di Sarconi” PGI (*Phaseolus vulgaris* L.)<sup>†</sup>

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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 resulted. Between paper test, specific for detecting the *C. lindemuthianum*, revealed the presence of this pathogen for the 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

## 1. Introduction

*Phaseolus vulgaris* L., known as 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 about 24.000 ha (FAOSTAT 2018. Statistical database 2018, Available at <http://faostat.fao.org>). 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 the germination, infect to death the 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.. The 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

**Samples 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 ( $25^{\circ} \pm 2^{\circ}\text{C}$ ) 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 to identify the spores of the fungi contaminating the seed surface [8,9]. For each test, 400 seeds (4 replicates  $\times$  100) were considered. The separation of spores and mycelium from the tegumental surface was obtained by stirring the seeds in presence of sterile, distilled water. After filtration, the suspension was centrifugated at  $5.000\times g$  for 11 min. The precipitated was resuspended in 200  $\mu\text{L}$  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 streptomycin (Sigma-Aldrich, Italy), and incubated at  $22^{\circ}\text{C}$  in the dark, for 5–8 days. Thenafter, 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  $\times$  100) were disinfected with a 1% sodium hypochlorite solution for 10 min and distributed on the moistened paper. The plates were incubated at  $20^{\circ}\text{C}$  for 10 days, 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  $\times$  50) were immersed for 10 min in a 1% sodium hypochlorite solution and then air-dried. Subsequently, 4 sub-samples 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 during incubation (7 days at  $20^{\circ}\text{C}$ ). Then, the seeds and cotyledons were observed, and black depressed areas with well defined contours searched. The typical signs of the antracnose 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, colour, shape and size of conidia, reproductive structures) were revealed under the optical microscope (Axioskop, Zeiss, Germany) with objectives  $20\times$ ,  $40\times$  and  $100\times$ . For the microscopical and morphological identification of the fungal isolates scientific literature, 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 (analysoft, 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 of the 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.

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

\*\*\* Pr < 0.001; \*\* Pr < 0.01; \* Pr < 0.05.

### 3.2. Isolation and Identification of Seed Mycoflora with Washing Test

The 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 were found to be 18, as reported in Table 2.

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.

**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    |         |                  |         |
|--------------------------------------|---------|---------|------------------|---------|---------|---------|------------------|---------|
|                                      | Ciuoto  |         | Cannellino Rosso |         | Ciuoto  |         | Cannellino Rosso |         |
| Locality                             | Sarconi | Paterno | Sarconi          | Paterno | Sarconi | Paterno | Sarconi          | Paterno |
| <b>Fungal microflora</b>             |         |         |                  |         |         |         |                  |         |
| <i>Alternaria</i> sp.                | +       | -       | -                | -       | -       | +       | +                | -       |
| <i>Alternaria alternata</i>          | -       | -       | -                | -       | -       | +       | -                | +       |
| <i>Aspergillus</i> sp.               | -       | -       | +                | +       | -       | -       | -                | -       |
| <i>Aspergillus flavus</i>            | -       | -       | +                | +       | +       | -       | -                | -       |
| <i>Aspergillus niger</i>             | +       | -       | -                | -       | -       | -       | +                | -       |
| <i>Cladosporium cladosporioides</i>  | -       | -       | -                | +       | +       | +       | +                | -       |
| <i>Botrytis</i> sp.                  | -       | -       | +                | -       | -       | +       | -                | +       |
| <i>Colletotrichum lindemuthianum</i> | -       | +       | -                | -       | -       | +       | -                | -       |
| <i>Fusarium oxysporum</i>            | -       | -       | -                | -       | -       | +       | -                | +       |
| <i>Fusarium solani</i>               | -       | +       | -                | -       | -       | +       | -                | -       |
| <i>Mucor hiemalis</i>                | +       | -       | -                | -       | -       | -       | +                | -       |
| <i>Penicillium</i> sp.               | -       | -       | +                | +       | -       | +       | +                | +       |
| <i>Penicillium expansum</i>          | -       | +       | -                | -       | -       | +       | -                | -       |



<sup>1</sup> The differences of rizzottoniosis from *Rhizoctonia solani* between untreated and treated seeds are significant for  $\chi^2 = 87.04^{***}$ , at  $p < 0.000$ ; <sup>2</sup> 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 are reported 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, all detected by between paper test.



**Figure 3.** (a) Acervulus of *Colletotrichum lindemuthianum* (similar to sporodochium) in vitro, with bristles in evidence (resolution 20 $\times$ ); (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, 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-emergence and post-emergence, 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., *Botrytis* sp., *Mucor* sp., *Rhizopus* 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*, *R. solani*.

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

Regarding 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 antrachnose 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 tegumental 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 rizzottoniosis 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 detect 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.

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