

Development of Integrated Disease Control Measures for the Valorisation of Traditional Crops in Southern Italy: The Case Study of *Fagioli di Sarconi*

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Abstract The cultivation of *Fagioli di Sarconi* (*FS*), a pool of traditional varieties protected with the mark PGI (Protected Geographical Indication) cultivated in the National Park of Agri Valley in Basilicata (southern Italy), is limited by common bacterial blight (CBB), caused by the seed-borne bacterium *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) and *X. a.* pv. *phaseoli* var. *fuscans* (*Xapf*), whose control is difficult because of the lack of safe bactericides. Hence, studies were undertaken to assess the susceptibility/tolerance of selected varieties toward the pathogens and to develop eco-compatible measures for the disease management. Five *FS* varieties showed a differential response to inoculations with virulent strains of the pathogens, and hence, two tolerant cultivars were selected for the introgression of CBB resistance characters. Among the main active components of some essential oils, eugenol determined a highly significant reduction of *Xap* density on bean seeds, though at the higher dosage, the seed germination reduction was observed. These data indicate eugenol as potentially useful for bean seed disinfection from *Xapf*, though further studies appear necessary. Among 162 bacterial isolates from bean rhizosphere, six caused a clear reduction of lesions size ranging from 30 to 66 % suggesting that induced systemic resistance may be involved in that feature.

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1 Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important legumes due to its commercial value and its high content of quality carbohydrates, proteins, minerals and vitamins. FAO data (FAO Statistics Division 2014) on bean production in Europe indicate that in 2012, Belarus, Spain and Italy were the main bean producers. In fact, bean production was 227.259 t in Belarus (only for dry beans), 175.300 t in Spain (165.400 for string and 9.900 for dry beans) and 145.933 t in Italy (134.124 for string and 11.809 for dry beans). In Italy, besides the commercial cultivars, there is an abundance of traditional varieties with unique nutritional features and for which there is a traditional market (Dinelli et al. 2006). This is the case of the *Fagioli di Sarconi* (FS), a pool of traditional high-value traditional varieties, selected from various landraces (Masi et al. 1999; Piergiovanni et al. 2000) and protected by the European Union (Reg. CEE No. 1263/96) with the mark PGI (Protected Geographical Indication).

FS are cultivated in the National Park of the Agri Valley in Basilicata (southern Italy) for dry seed production (Brandi et al. 1998). The germplasm, preserved on farm by the *Consorzio di Tutela dei Fagioli di Sarconi*, has been extensively characterised for its biochemical and nutraceutical traits (Piergiovanni et al. 2000; Lioi et al. 2005; Dinelli et al. 2006) but nothing is known about its response to diseases. As a matter of fact, the above varieties are plagued by the common bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Dye (*Xap*) and *X. a.* pv. *phaseoli* var. *fuscans* (*Xapf*) (Vauterin et al. 1995), and since 2002, there have been several severe outbreaks of this disease. Bean field surveys in 2001–2002 showed that at the end of the production cycle, nearly 100 % of the plants were infected, with the consequent heavy crop loss. The disease is endemic and some evidences indicate its introduction by long time. The use of bean seed grown on farm, possibly infected and/or contaminated by the pathogens, the limited copper sprays and the use of overhead irrigation have facilitated the dissemination of the pathogen and the maintenance of a high level of the inoculum potential. Year by year, bacterial disease outbreaks have been greatly facilitated by the particular climatic conditions which, as observed in the above years, were characterised by heavy rains during the periods from July to September (Lo Cantore et al. 2004a). CBB pathogens infect all the plant organs and the disease development is favoured by warm temperature (25–35 °C) and humid conditions (Gilbertson and Maxwell 1992; Saettler 1989). CBB symptoms on foliage are water-soaked spots that enlarge forming dark brown necrotic lesions often surrounded by chlorotic zones. Infected pods exhibit circular water-soaked areas that turn to reddish-brown lesions. Pod infection often causes discoloration, shrivelling and bacterial contamination/infection of seeds though in some cases, they may appear healthy (Saettler 1989).

The causal agents of CBB, *Xap* and *Xapf*, are distinguished from each other only because *Xapf* produces a brown pigment when grown on some specific agar media (Schaad et al. 2001). Nevertheless, there is considerable genetic diversity between *Xap* and *Xapf* (Chan and Goodwin 1999; Mkandawire et al. 2004; López et al. 2006; Mahuku et al. 2006), as recent AFLP analyses have confirmed (Lo Cantore and Iacobellis 2007; Alavi et al. 2008; Lo Cantore et al. 2010b). For that, a revision of the two pathogens classification has been proposed (Schaad et al. 2005, 2006).

Dissemination in the field is mainly determined by wind-driven rain and overhead irrigation, but also by insects, field workers and contaminated equipment (Gilbertson and Maxwell 1992; Saettler 1989), which may play an important role. Common bacterial blight is the major seed-borne disease of common bean worldwide (Tarlan et al. 2001; Miklas et al. 2003), and the best way to manage CBB includes the use of pathogen-free seed (Zanatta et al. 2007). In order to control CBB, the use of bean cultivars with genetic tolerance/resistance to the disease is the most practical method (Coyne and Schuster 1974; Yoshii et al. 1978) though the availability of CBB-resistant cultivars is limited. In most of the cases, the resistance/tolerance of traditional varieties to CBB is unknown. This is the case of *FS* varieties. Furthermore, pathogen-free seed is a prerequisite for a healthy crop, but to date no efficient disinfection method is available (Lo Cantore et al. 2009). Antibiotics, in fact, are actually restricted or forbidden in the agricultural practices in many countries (McManus et al. 2002) and the use of copper compounds, because of their general toxicity and impact on the environment, is constrained in Europe (EU rule no. 473/2002). Furthermore, chemical disinfectants such as chlorine, inorganic acids, organic acids and heat treatments have been used for disinfection of potentially contaminated seeds surface or to cure infected seeds, but seed devitalisation has been reported (Claffin 2003).

The above consideration prompts the need to assess, first, the response of the selected *FS* bean varieties to CBB with the final aims to select resistant/tolerant traditional varieties and to introgress tolerance/resistance characters into the *FS* varieties of interest. Moreover, of interest was the development of alternative methods for the control of CBB to be used in integrated crop management as well as in bio-organic agriculture. Several studies have pointed out the possibility to use essential oils and/or their components in medical and plant pathology as well as in the food industry for the control of microorganisms pathogenic to consumers and/or responsible for food spoilage (Seow et al. 2014). Nevertheless, most of the studies are mainly focused on the *in vitro* assessment of the antimicrobial activity (Si et al. 2006; Terzi et al. 2007), and the exploitation of essential oils for the control of plant diseases is still in its infancy (Tinivella et al. 2009; Kotan et al. 2010). Another opportunity for plant defence towards diseases is the use of beneficial bacteria inhabiting plant rhizosphere. In several works, it was demonstrated that some bacteria belonging to *Pseudomonas* and *Bacillus* genera are able to provide different mechanisms (direct or plant mediated) for suppressing plant diseases (Saharan and Nehra 2011).

In this chapter, we report some of the results obtained in studies aimed to develop methods for integrated control of CBB.

2 Response of *Fagioli di Sarconi* Varieties to CBB

The objective of the present work was to evaluate the response to CBB of five traditional bean varieties (*Tondino bianco*, *Verdolino*, *Cannellino*, *Tabacchino* and *Ciuoto*) of the *FS* variety pool, in comparison to four available resistant bean breeding lines (VAX-4, USDK-CBB-15, ABC-Wiehing and USCR-CBB-20) (Singh et al. 2001; Miklas et al. 2006, 2011; Mutlu et al. 2008). Aliquots of bacterial suspensions of the highly virulent *Xap* and *Xapf* strains were inoculated into the first trifoliate mesophyll of bean plants (Lo Cantore et al. 2010a).

The traditional varieties *Tondino bianco*, *Cannellino*, *Verdolino*, *Ciuoto* and *Tabacchino* showed the typical CBB symptoms, and, in particular, at the inoculation sites, tiny water-soaked lesions 14 days after inoculation which then expanded and turned into necrotic spots surrounded by chlorotic halos were observed (see Fig. 1a, c). As expected, necrotic lesions of the hypersensitive reaction were observed on the CBB-resistant breeding lines (see Fig. 1b, c).

Twenty-eight days after inoculation, the *FS* varieties statistically differed in their susceptibility depending on the pathogen strain used (see Fig. 2). Indeed, while strain USB749 (ICMP14929) of *Xapf* caused on *Tondino bianco*, *Cannellino* and *Verdolino* lesions significantly larger ($P < 0.0001$; LSD.05 = 5.14 mm) than those ones on cv. *Ciuoto* and *Tabacchino* (see Fig. 2a), *Xap* USB771 (ICMP14932) caused lesions on *Tondino bianco* and *Tabacchino* significantly larger ($P < 0.001$; LSD.05 = 1.82 mm) than those on *Verdolino*, *Ciuoto* and *Cannellino* (see Fig. 2b). The lesions on *Ciuoto* and *Cannellino* did not differ significantly from the lesions

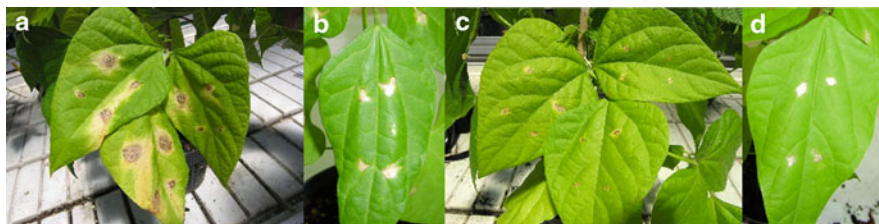


Fig. 1 Symptoms on the traditional variety *Verdolino* (a–c) of *Fagioli di Sarconi* and hypersensitive necrotic lesions on the CBB-resistant breeding line ABC-Wiehing (b–d) 21 days after syringe infiltration with 10^8 CFU ml^{-1} suspensions of the highly virulent strain USB749 (ICMP14929) of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (a–b) and of strain USB771 (ICMP14932) of *X. a.* pv. *phaseoli* (c–d). Adapted with permission from Lo Cantore P, Figliuolo G, Iacobellis NS (2010) Response of traditional cultivars of *Fagioli di Sarconi* beans to artificial inoculation with common bacterial blight agents. *Phytopathol Mediterr* 49:89–94. Copyright 2010 Firenze University Press

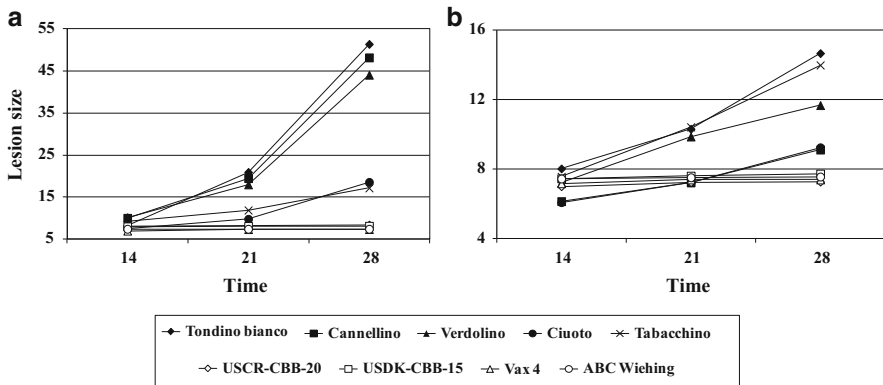


Fig. 2 Response given by the traditional bean varieties of the *Fagioli di Sarconi* and by CBB-resistant breeding lines 28 days after syringe infiltration of 10^8 CFU ml^{-1} suspensions of the highly virulent strain USB749 (ICMP14929) of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (a) and of strain USB771 (ICMP14932) of *X. a. pv. phaseoli* (b). Adapted with permission from Lo Cantore P, Figliuolo G, Iacobellis NS (2010) Response of traditional cultivars of *Fagioli di Sarconi* beans to artificial inoculation with common bacterial blight agents. *Phytopathol Mediterr* 49:89–94. Copyright 2010 Firenze University Press

on the breeding lines USDK-CBB-15, ABC-Wiehing and VAX-4, but they significantly differed from those on USCR-CBB-20 (see Fig. 2b).

In conclusion, the findings that the varieties *Ciuto*, *Tabacchino* and *Cannellino* appeared to be less susceptible or tolerant to CBB are positive since it is well established that the use of pathogen-tolerant germplasm, rather than resistant one, is advisable in order to avoid the selection of the pathogen population overcoming the resistance. The above varieties, apart from their agronomic, biochemical and nutraceutical traits, appear good candidates for the introgression of CBB resistance characters in a breeding programme.

The results further suggest that the inoculation procedure is suitable to evaluate the susceptibility/tolerance and the resistance of bean cultivars and/or breeding lines. However, the response of the traditional bean varieties to CBB pathogens inoculation needs to be further confirmed by using different plant parts (i.e. pods) since the degree of resistance/susceptibility of *Phaseolus* spp. depends on the plant organ (Aggour et al. 1989; Rodrigues et al. 1999; Marquez et al. 2007).

The development of new bean breeding lines genetically resistant to *Xapf* was another aim of this work. The selection applied to these breeding lines should tend to combine, in their genetic background, the gene of resistance and all agronomic and nutraceutical traits present in the old varieties. The bean traditional varieties *Tabacchino* and *Ciuto*, and bean breeding lines VAX-4 and USCR-CBB-20 carrying the resistance to *Xaf* (Singh et al. 2001; Miklas et al. 2011), were used in the breeding plan.

Numerous ♀*Ciuto* × USCR-CBB-20♂, ♀*Ciuto* × VAX-4♂, ♀*Tabacchino* × USCR-CBB-20♂ and ♀*Tabacchino* × VAX-4♂ crosses and related reciprocals were made in greenhouse. All F_1 plants were grown in the field until the production

of eight groups of F_2 seed progenies. F_3 seeds obtained from F_2 plants selected for *Xapf* resistance were sown in the field and related plants submitted to agronomic selection/evaluation. The choice of the best single plants was based on production capacity, plant hardness and health, and pod and seed quality. Only three types (groups) of crosses, out of the eight performed ($\text{♀Ciuoto} \times \text{VAX-4♂}$, $\text{♀Tabacchino} \times \text{VAX-4♂}$, $\text{♀Tabacchino} \times \text{USCR-CBB-20♂}$), produced progenies with good plant and seed traits. The seed progenies are at F_4 generation level.

3 *Fagioli di Sarconi* Seed Disinfection

Our previous studies showed the antibacterial activity of coriander, caraway and cumin essential oils towards 29 bacterial pathogens of plants, including strains of *Xap* and *Xapf* (Iacobellis et al. 2005; Lo Cantore et al. 2004b). The objective of this study was to evaluate the *in vitro* antibacterial activity of the 19 main components of the above essential oils and to assess their potential use for bean seed disinfection. In disc diffusion assay (Lo Cantore et al. 2009), terpenoids and phenylpropanoids, having phenol and alcohol functionalities, showed a high bactericidal activity, inhibiting the growth of all bacterial strains used in this study, whereas a lower activity was shown by monoterpenes containing ketone, aldehyde and ester functionalities and the phenylpropanoids anethol. Monoterpenoids and sesquiterpenoid caryophyllene showed a lower activity and only on a limited number of the target bacteria. Based on the above results, eugenol was chosen in order to evaluate its disinfecting effects on the *FS* variety *Ciuoto* seeds artificially contaminated with *Xapf* (Lo Cantore et al. 2009). Eugenol seed treatments (1, 2, 4 and 8 mg ml⁻¹) caused a statistically significant reduction ($P < 0.0001$) of the bacterial population on bean seeds relative to the control sample (see Fig. 3). Assays with different bacterial densities bearing seeds and treatments with 4 mg ml⁻¹ eugenol emulsions confirmed the higher efficiency of this essential oil to reduce bacterial population densities on bean seed surface when compared to the tetracycline treatments at a comparable MIQ value (100 µg ml⁻¹). However, eugenol emulsions at concentration equal or superior to 2 mg ml⁻¹ caused a significant reduction of the seed germination compared to the control (see Fig. 4).

In conclusion, essential oils and/or pure components appear to be good bactericides as alternatives to antibiotics for the control of seed-borne plant pathogen bacteria.

The use of eugenol instead of complex oil mixtures may be desirable since this avoid the variability of essential oils and, furthermore, the possible toxicity of other no bactericide essential oil components. The reduction of bean seed germination after treatments with eugenol was already reported (Asplund 1968; Reynolds 1987; Oosterhaven et al. 1995), but the limited effect on bean seed germination observed at eugenol lower concentrations indicates a starting point for a possible amelioration of the disinfectant formulation and disinfection method.

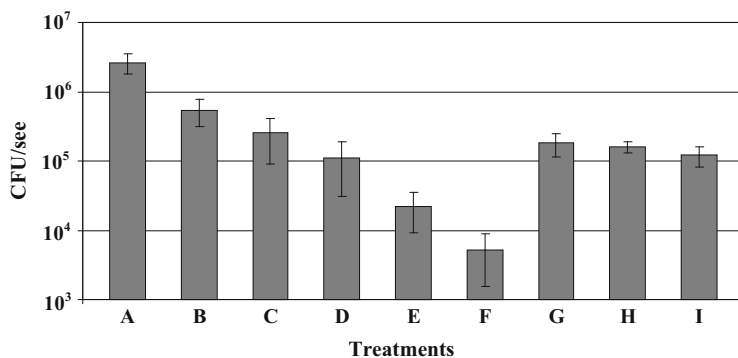


Fig. 3 Bacterial populations of the spontaneous mutant resistant to rifampicin of strain ICMP239 of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* on bean seeds bearing about 2.6×10^6 CFU per seed after different treatments (A = control, no treated seeds; B = control, seeds treated with water containing 0.01 % Tween 20; C, D, E and F = seeds treated with 1, 2, 4 and 8 mg ml⁻¹ of eugenol in water emulsions containing 0.01 % Tween 20, respectively; G, H and I = seeds treated with 50, 100 and 200 µg ml⁻¹ tetracycline solutions containing 0.01 % Tween 20, respectively). Bars on the columns correspond to the standard error of the mean. Means of bacterial populations on bean seeds after eugenol and tetracycline treatments, evaluated by the *t*-test in comparison with the means of bacterial populations on bean seeds of the controls A and B, are statistically different ($P \leq 0.002$). Adapted with permission from 'Lo Cantore P, Shanmungaiah V, Iacobellis NS (2009) Antibacterial activity of essential oil components and their potential use in seed disinfection. *J Agric Food Chem* 57:9454–9461'. Copyright 2009 American Chemical Society

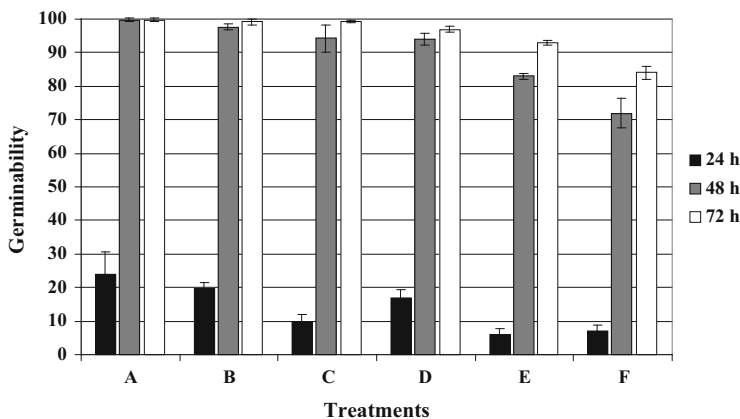


Fig. 4 Germination of bean seeds after different treatments with eugenol (A = control, no treated seeds; B = control, seeds treated with water containing 0.01 % Tween 20; C, D, E and F = seeds treated with 1, 2, 4 and 8 mg ml⁻¹ of eugenol in water containing 0.01 % Tween 20, respectively). Bars on the columns correspond to the standard error of the mean. The *t*-test of data at 72 h showed that means of germinated seeds after eugenol treatments (8, 4 and 2 mg ml⁻¹), in comparison with the controls (A and B), are statistically different ($P < 0.0001$) and ($P < 0.02$), respectively. No statistical differences were observed in the case of treatments with 1 mg ml⁻¹ eugenol emulsion. Adapted with permission from 'Lo Cantore P, Shanmungaiah V, Iacobellis NS (2009) Antibacterial activity of essential oil components and their potential use in seed disinfection. *J Agric Food Chem* 57:9454–9461'. Copyright 2009 American Chemical Society

4 Beneficial Rhizobacteria as Biocontrol Agents of CBB in *Fagioli di Sarconi*

Rhizobacteria are microorganisms living plant rhizosphere and some of them are able to reduce plant diseases by several direct mechanisms (i.e. niche exclusion, competition for nutrients, siderophore-mediated competition for iron, antibiosis and production of extracellular hydrolytic enzymes) (Martinez-Viveros et al. 2010) or indirectly by stimulating the basal plant defence mechanisms, phenomenon called induced systemic resistance (ISR) (Van Loon 2007).

As already discussed, CBB is difficult to manage via the conventional methods. In an integrated view of CBB control, the biological one based on the use of antagonist rhizobacteria may represent another opportunity.

The aim of this study was to evaluate the potential of selected rhizobacteria isolated from bean rhizosphere to protect plants against CBB in *in vitro* and greenhouse conditions. In total, 162 rhizobacteria were isolated from bean rhizosphere in the National Park of Agri Valley (Basilicata, southern Italy) and screened in dual-plate assays for their potential capacity to antagonise the growth of CBB pathogens. Sixty out of 162 rhizobacteria inhibited *in vitro* the growth of *Xap*, and 38 of these were also active against *Xapf*; thus, they were assayed for hydrolytic enzyme production. Subsequently, they were evaluated, when applied to seeds before sowing, for their possible effect on *Xapf*-plant interactions. The results of these assays have allowed selecting six bacterial isolates that were capable to protect, with diverse efficacy, bean plants artificially inoculated, *in vitro* and greenhouse assays, with a highly virulent strain of the mentioned pathogen. Indeed, the reduction of lesions size ranged from about 25 to 55 % *in vitro* and from about 35 to 65 % in greenhouse experiments compared to the controls (see Fig. 5). The six selected isolates were identified by partial PCR amplification of 16S rDNA as three strains of *Pseudomonas brassicacearum* subsp. *brassicacearum*, two strains of

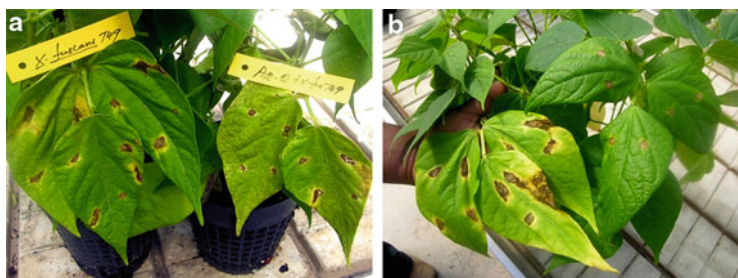


Fig. 5 Symptoms of common bacterial blight on trifoliate leaves developed 21 days after syringe without needle inoculation with suspension (10^8 CFU ml^{-1}) of strain USB749 (ICPPM 14929) of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*. Comparison between bean plants obtained from not bacterised (left) and bacterised seeds (right). In (a) and (b), bean plants from seeds are treated with bacterial suspensions (10^8 CFU ml^{-1}) of *Pseudomonas brassicacearum* subsp. *brassicacearum* strains (USB2101, USB2102)

P. putida and one of *Bacillus megaterium*. The antagonist bacteria were then further characterised for phosphate solubilisation (Nautiyal 1999), nitrogen fixing activity (San Yu et al. 2011), production of indole compounds (Gordon and Weber 1951) and siderophores (Schwyn and Neilands 1987), haemolytic capacity (Lo Cantore et al. 2006) and production of ammonia (Cappuccino and Sherman 2010) and of hydrogen cyanide (Lorck 1948). Finally, bacteria were evaluated for adaptability to salinity, pH and temperature gradients (Giorgio 2014; Giorgio et al. 2013).

The mechanisms underlying bean plant protection are not completely understood so far. At the base of the pathogen control exerted by the six mentioned bacteria, some likely explanations exist. In the pathogenicity assays, the rhizobacteria and the challenging pathogen were apparently spatially separated and this may suggest that the observed effect may result from the activation in bean plants of induced systemic resistance (ISR) (Van Loon 2007). The ability to trigger ISR by some of the above rhizobacteria has been ascertained in further parallel studies in the pathosystem *Arabidopsis thaliana*-*X. campestris* pv. *amoriaceae* (Giorgio 2014; Giorgio et al. 2013). Molecular studies leading to determine the occurrence of ISR in bean-*Xapf* pathosystem are in progress. However, a direct effect of rhizobacteria on the pathogen at the infection site cannot be excluded since it has been reported that bacteria applied to soil or on seeds colonise rhizosphere and then may move up to the phylloplane (Nautiyal et al. 2002) or to behave as either facultative or opportunistic endophytes (Hardoim et al. 2008). Some characters shown by rhizobacteria under study may be the responsible of the possible direct antagonism exerted on the pathogens. In fact, the haemolytic activity shown by these bacteria may depend on the production of antimicrobial substances affecting biological membranes which is a quite common feature in plant-associated *Pseudomonas* spp. and *Bacillus* spp. (Ongena and Jacques 2008; Raaijmakers and Mazzola 2012). Furthermore, of great interest is the fact that strains of *P. b.* subsp. *brassicacearum* produce HCN reported as inhibitor of cytochrome C oxidase in the respiratory chain (Knowles 1976) and binding metalloenzymes (Blumer and Haas 2000) determining deleterious effect in microorganisms inhabiting the same ecological niche and nonproducers of HCN. On the other side, *P. putida* strains appeared to produce ammonia that accomplishes several biological roles and, in particular, its toxicity is well known (Weise et al. 2013). The six antagonistic rhizobacteria showed also desirable plant growth-promoting ability since they can supply inorganic phosphate to plants (Schachtman et al. 1998) via phosphatase activity which leads to the production of organic acids that decrease soil pH (Rashid et al. 2004), so that this may limit some soilborne phytopathogenic organisms, as demonstrated in the case of *P. ultimum* and *F. oxysporum* (Alhussien 2012). Moreover, in this regard, important is the fact that the six rhizobacteria produce also indole compounds that promote bacterial environmental adaptation during stress conditions such as UV, salt and acidity (Bianco et al. 2006). Finally, their siderophore production may contribute to plant growth-promoting activity by depriving pathogens of iron accomplishing, in this way, their antagonistic activity (Kloepper et al. 1980). In conclusion, the fact that numerous mechanisms may be involved in leading to the observed effects makes worthwhile a deeper investigation on the system considered.

5 Concluding Remarks

In conclusion, the findings that the *Fagioli di Sarconi* traditional varieties *Ciuoto*, *Tabacchino* and *Cannellino* appeared to be less susceptible or tolerant to CBB are positive since it is well established that the use of pathogen-tolerant germplasm, rather than resistant one, is advisable in order to avoid the selection of the pathogen population, leading to the resistance being overcome. The introgression and stabilisation of CBB resistance characters into some of the above tolerant traditional varieties, in the breeding programme still in progress, may bring about to highly agronomic and nutraceutical values varieties with potential economic impact.

Essential oils and/or pure components such as eugenol appear to be good bactericides as alternatives to antibiotics for the sanitation of bean seeds from CBB pathogens as well as for the control of other seed-borne plant bacterial pathogens. However, studies either on formulations or on the application method of eugenol appear necessary to avoid undesirable toxic effect on seeds and plantlets.

Lastly, of highly value is the finding that strains of *Pseudomonas brassicacearum* subsp. *brassicacearum*, *P. putida* and *Bacillus megaterium*, isolated from bean rhizosphere, are capable of protecting bean plants artificially inoculated with the CBB pathogen. Also in these cases, further studies appear necessary to transfer these potential antagonists in the agriculture practices.

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