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Research Article

In vitro Antagonism of Rhizobacteria Isolated from *Coffea arabica* L. against Emerging Fungal Coffee Pathogens

In vitro antagonistic effects of rhizobacteria associated with *Coffea arabica L.* against some fungal coffee pathogens were studied. The aims were to screen indigenous coffee-associated isolates for their inherent antagonistic potential against major coffee wilt diseases induced by *Fusarium* spp. Antagonistic effects, siderophore, HCN and lytic enzyme production were determined on standard solid media. Chemical methods were employed to categorize the major types of siderophores. From a total of 212 rhizobacterial isolates tested, over 10 % (all *Pseudomonas* and *Bacillus* spp.) exhibited remarkable inhibition against *Fusarium* spp. One isolate AUPB24 (*P. chlororaphis*) showed maximum inhibition of mycelial growth against all fungal pathogens tested, whereas other isolates were mostly inhibitory to *F. stilboides* and *F. oxysporum*. The isolate AUBB20 (*B. subtilis*) was most antagonistic to *F. xylarioides*. Of the rhizobacterial isolates tested, 67 % produced siderophores and 35 % produced HCN. Many strains (all *Pseudomonas* spp.) produced siderophores of the hydroxamate type and only a small proportion produced those of the catecholate type. Few antagonists showed chitinase activity. The production of siderophores and HCN by *Pseudomonas* spp., lipase and protease by all antagonists and β-1,3-glucanase by several *Bacillus* spp. could be considered the major mechanisms involved in the inhibition of fungal growth. The in vitro results provide the first evidence of an antagonistic effect of coffee-associated rhizobacteria against the emerging fungal coffee pathogens *F. stilboides* and *F. xylarioides* and indicate the potential of both bacterial groups for biological control of coffee wilt diseases.

Keywords: Fungal coffee pathogens, Rhizobacteria, Siderophores

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1 Introduction

Ethiopia is the primary gene center for Arabica coffee *(Coffea arabica* L.) and coffee is the major agricultural export product [1]. Coffee is grown by smallholders under various kinds of shade trees [2, 3].

African coffee farmers, however, are currently facing many difficulties, e.g. yield losses, due to emerging serious fungal wilt diseases [4–6]. Coffee wilt disease or tracheomycosis caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) is becoming important in some regions of Eastern, Central and West Africa, not only in Robusta but also in Arabica coffee [4, 5, 7]. The disease severely attacks the vascular system of the plant, causing wilting and eventually die-back [8]. Coffee vascular disease (tracheomycosis) was documented in Ethiopia for the first time in the 1970s [4] and in 1993 in Uganda, where it is causing significant yield losses in Robusta coffee [5]. In major Arabica coffee growing areas of southwestern Ethiopia, including Bebeka, Teppi, Jimma and Gera, the incidence of *Fusarium* (*Gibberella*) *xylarioides* is reported to be 60 % and the fungus is causing significant yield losses due to very severe damage and death of millions of trees [4]. Those authors suggest that more aggressive strains of the pathogen may have developed. *Fusarium stilboides* Wollenw. (telemorph: *Gibberella stilboides*), the causal agent of coffee bark disease, is also

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present in some African countries, particularly in Ethiopia, Kenya, Malawi and Tanzania [7]. Its characteristic symptom is a scaling of the bark, leading to stem cankers and a progressive die-back of the whole tree. Furthermore, the phytopathogenic fungus *Fusarium oxysporum* Schlechtend.:Fr. causes destructive vascular wilts in a wide variety of crops including coffee [9]. A report from Uganda [6] indicates that *F. xylarioides* causes more deaths of young coffee plants than any of the other *Fusarium* species, emphasizing the severity of this pathogen.

At present, no appropriate control measures are in place against these emerging coffee fungal pathogens. The high cost of pesticides, the appearance of fungicide-resistant pathogen biotypes and other social and health-related impacts of conventional agriculture on the environment have increased interest in agricultural sustainability and biodiversity [10]. Thus, millions of coffee farmers in developing countries are facing problems not only with low coffee prices but also a growing interest in organically-grown coffee.

The combination of these matters necessitates eco-friendly control methods, e.g. biological controls, which have been studied as an alternative or complementary approach to physical and chemical disease control measures for over seven decades [11]. Current developments in sustainability involve a rational exploitation of soil microbial activities affecting plant development [12]. Among the plant growth-promoting rhizobacteria (PGPR), *Pseudomonas* and *Bacillus* spp. in particular have been utilized widely in biocontrol methods in low input agricultural production systems [13].

Bacterial antagonism towards plant pathogenic fungi involves a diverse array of mechanisms such as the production of antibiotics [14], siderophores [15], lytic (fungal cell wall degrading) enzymes [16] and hydrogen cyanide (HCN) [17], competition for nutrients and parasitism [18], as well as emission of potent antifungal volatile organic compounds [19]. Siderophores are produced by a diverse array of microorganisms and vary widely in their overall structure, but most of them contain hydroxamate or catecholate groups [20].

Little is known about microorganisms associated with *Coffea arabica* regarding their antagonistic effect against coffee wilt diseases caused by noxious *Fusarium* spp. However, reports have revealed close associations of useful microorganisms with *Coffea arabica* including *Bacillus*, *Burkholderia*, *Pseudomonas*, members of the Enterobacteriaceae and others [21, 22].

Therefore the authors considered it advantageous to screen indigenous beneficial bacterial antagonists from the homeland (center of origin) of *Coffea arabica* where both pathogens and antagonists are expected to display wide abundance and biodiversity.

Therefore, our goals were to screen native coffee-associated rhizobacterial antagonists from our own collection against some important fungal coffee pathogens and to evaluate the underlying mechanisms by studying their ability to produce siderophores, HCN and lytic enzymes. In addition, the main types of siderophores produced were assessed.

2 Materials and Methods

2.1 Origin of Rhizobacterial Isolates

The rhizobacterial isolates used (see below) originated from natural coffee forests at two locations in southwestern Ethiopia: *i)* Bonga district (Kaffa zone) in Southern Nations and Nationalities Peoples' Regional State (S.N.N.P.R.S); and *ii)* Yayu district (Illubabor zone) in Oromia Regional State. The sites are located between 07°28'-08°28'N and 35°50'-36°45'E, with an altitude range of 1376–1890 m asl.

In general, southwestern Ethiopia has diurnal and seasonal fluctuations in temperature (14 to 30 °C), relative humidity (43 to 85 %) and heavy rainfall (1000 to 2000 mm per year) with infrequent rain over eight months [23]. All these factors together with good shade [3] could create conditions conducive to growth for noxious fungal pathogens.

The bacterial isolates were from our own culture collection in Addis Ababa University, Department of Biology, Addis Ababa, Ethiopia. They were originally isolated from rhizospheres of *Coffea arabica* growing in the above natural forests. These isolates have been morphologically, biochemically and physiologically tested and identified using API kits and 16S rDNA analysis (Muleta et al. unpubl.).

2.2 Differentiation of Fluorescent Pseudomonads

To differentiate between fluorescent and non-fluorescent pseudomonads, young cultures were inoculated on King's B medium [24]. After incubation at 30 °C for 48 h, fluorescent colonies were checked under UV light (Chromato-UVE, Ultraviolet products, Inc., San Gabriel, California, USA) at 365 nm.

2.3 Cyanogenesis and Siderophore Production

Cyanide and siderophore productions were detected qualitatively as described by Bakker & Schippers [25] and Schwyn & Neilands [26], respectively. To determine the type of the siderophore, culture supernatants were used. The presence of catecholate type siderophores was tested according to Arnow [27], using 2,3-dihydroxybenzoic acid as the standard. Similarly, the presence of hydroxamate siderophores was checked according to Atkin et al. [28], using desferal (Novartis International AG, CH-4002 Basel, Switzerland) as a positive control. The absorption spectra of the culture supernatants were measured in the visible range using a Shimadzu UV-VIS spectrophotometer (model UV-1601) at 500 (Arnow's method) and 480 nm (Atkin's method), respectively.

2.4 Fungal Isolates and Culture Conditions

Fungal isolates (*F. oxysporum*, *F. stilboides* and *F. xylarioides*) were kindly provided by Dr. David M. Geiser, Director, Fusarium Research Center, Department of Plant Pathology, Pennsylvania State University, USA. The isolates (see Tab. 1) were

Table 1. Accession number, origin and source/host of the fungal coffee pathogens kindly provided by Dr. Geiser (see Materials and Methods).

Accession number	Species	Host/substrate	Geographic origin
FRC O-1206	*Fusarium oxysporum*	Coffee seedling stem base	Papua New Guinea
FRC L-0069	*Fusarium stilboides*	*Coffea arabica* berries	Rhodesia Coffee Research Station
FRC L-0102	*Fusarium xylarioides*	*Coffea robusta*	Guinea

maintained on synthetic low-nutrient agar (SNA) [29] slants at 4 °C for the subsequent activities.

2.5 Suitability of the Media for Antagonistic Studies

The suitability of different media for the simultaneous growth of fungal pathogens and the rhizobacterial isolates (antagonists) was checked (see Tab. 2).

Table 2. Suitability of different media for simultaneous growth of both fungal pathogens and the bacterial isolates for antagonistic studies.

Culture	Medium				
	PDAFS	PDAHS	KBFS	KBHS	SNA
F. oxysporum	ND	++++	+++	+++	++++
F. stilboides	ND	+++	++	++	++
F. xylarioides	+	+	+	+	+++
Gram-negative isolates	+	+	ND	+++	±
Gram-positive isolates	±	±	+++	++	±

ND = not determined, PDAFS = potato dextrose agar full strength, PDAHS = potato dextrose agar half strength, KBFS = King's B medium full strength, KBHS = King's B medium half strength, SNA = synthetic low-nutrient agar, ± = almost no growth, + = growth, ++ = good growth, +++ = very good growth, ++++ = highly profuse growth.

2.6 Screening of Antagonism

For initial screening, a small fungal agar block (1 × 2 cm^2), from the leading margin of cultures propagated on potato dextrose agar for five to seven days at 25 °C was centrally placed on presolidified half-strength King's B medium (KBHS). Exponentially grown (24-h-old) bacterial cultures (two isolates/plate) were streaked as a broad band (making a straight short bar) approx. 3 cm away from the mycelial block at two opposite edges of duplicate Petri dishes (90 mm diameter). Plates were incubated at 25 °C for 7–10 days and potent rhizobacterial isolates selected depending on their degree of inhibition. A test was considered positive when bacteria interfered with the normal spread of the fungal mycelia on dual culture in two replications. In all antagonistic studies, a medium inoculated only with a test fungus served as a control. The SPSS statistic program was performed for experiments involving calculations and the significance was evaluated by Tukey's test ($p < 0.05$).

Rhizobacterial isolates that showed mycelial growth inhibition on dual culture medium were further tested for quantification of percentage fungal radial growth inhibition using the same medium, growth conditions and procedures. However, in this case a single bacterial antagonist was used per duplicate dual culture. Fungal radial growth inhibition (a clear zone between the edges of fungal mycelia and bacterial colonies) was calculated [30] after 5–7 days of incubation for the fast growing *F. oxysporum* and after 10 days for the slow growing *F. stilboides*. *F. xylarioides* was allowed to propagate in potato dextrose broth for five days at 25 °C. Similarly, bacterial cultures were grown on nutrient agar (Oxoid) for 48 h at 30 °C. Pre-solidified half-strength King's B medium was seeded with a fungal suspension (10^5 spores/mL) in duplicate and a bacterial agar block (1 × 1.5 cm^2) was placed at the center of the inoculated medium. The inhibition zone was measured (cm) after incubation at 25 °C for 10 days. Inhibition was clearly discerned by limited growth or the complete absence of fungal mycelium in the inhibition zone surrounding a bacterial colony.

2.7 Evaluation of Antifungal Enzymes

Lipolytic activity was assessed on lipase medium and proteolytic activity on skimmed milk agar [31]. Chitinase production was determined using purified fine chitin powder (2 g/L; Sigma) and β-1,3-glucanase production using β-1,3-glucan (5 g/L; laminarin, Sigma), following standard methods [32].

3 Results and Discussion

3.1 Fluorescence Production

Out of 67 coffee-associated rhizobacterial isolates tested, over 80 % of them showed intensive fluorescent pigments on King's B medium (see Tab. 3). Eleven isolates showed ambiguous results and one isolate was characterized as a nonfluorescent species although the API kit had identified it as *Pseudomonas fluorescens* (see Tab. 3).

3.2 Cyanide Production

Cyanide production of 67 rhizobacterial isolates was detected qualitatively. Over 50 % of the rhizobacteria isolates tested positive and belonged mainly to *P. fluorescens* (n = 34; see Tab. 3). This proportion is far higher than that reported from the rhizosphere of other plants by Kremer & Souissi [33] and Donate-Correa et al. [34], but similar to that reported by Bakker & Schippers [25]. In general, cyanogenesis was frequently found in members of the pseudomonads, which is corroborated by our findings.

Table 3. HCN and siderophore production by rhizobacterial isolates associated with *Coffea arabica* in natural forests, southwestern Ethiopia.

Isolate	Identity (API kit and 16S rDNA analysis)[a]	HCN	Flu.	Sid.	DYZ [cm]	Siderophore type	
						Cat.	Hy.
AUPB24	*P. chlororaphis* [b]	+	+	+	3.9	–	+
AUPB22	*P. chlororaphis* [c]	+	+	+	3.4	–	+
AUPB23	*P. chlororaphis* [b]	+	+	+	2.8	–	+
AUPB21	*P. chlororaphis* [c]	+	+	+	2.5	–	+
AUPY42	*Pseudomonas* sp.[b]	–	+	+	2.3	–	+
AUPY17	*Pseudomonas* sp.[b]	+	±	+	2.2	–	+
AUPB56	*P. fluorescens*	+	+	+	2.2	–	+
AUPB01	*Pseudomonas* sp.[b]	+	+	+	2.0	–	+
AUPB57	*P. fluorescens*	+	+	+	2.0	–	+
AUPB58	*P. fluorescens*	+	+	+	2.0	–	–
AUPB59	*P. fluorescens*	+	+	+	2.0	–	+
AUPY60	*P. fluorescens*	+	+	+	2.0	–	+
AUPY43	*Pseudomonas* sp.[b]	+	+	+	2.0	+	–
AUPB25	*Pseudomonas* sp.[b]	–	+	+	1.9	–	+
AUPY61	*P. fluorescens*	+	+	+	1.9	–	–
AUPB62	*P. fluorescens*	+	+	+	1.8	–	+
AUPB03	*Pseudomonas* sp.[b]	+	+	+	1.8	–	+
AUPB63	*P. fluorescens*	–	+	+	1.8	–	–
AUPY18	*P. fluorescens*	+	+	+	1.8	–	+
AUPB64	*P. fluorescens*	–	+	+	1.7	–	+
AUPB08	*Pseudomonas* sp.[b]	–	+	+	1.7	–	+
AUPB50	*Pseudomonas* sp.[b]	+	+	+	1.6	+	–
AUPB65	*P. fluorescens*	–	+	+	1.6	–	–
AUPY66	*P. fluorescens*	–	+	+	1.6	–	+
AUPY67	*P. putida*	–	+	+	1.6	–	+
AUPB68	*P. fluorescens*	+	±	+	1.5	–	+
AUPY41	*Pseudomonas* sp.[b]	–	±	+	1.5	+	–
AUPY69	*P. putida*	–	±	+	1.5	–	+
AUPB70	*P. putida*	–	+	+	1.4	+	–
AUSB14	*S. marcescens* [c]	–	–	+	1.4	+	–
AUPB71	*P. putida*	–	+	+	1.4	–	–
AUPY72	*P. fluorescens*	+	±	+	1.4	–	+
AUPB73	*P. fluorescens*	–	+	+	1.3	–	–
AUPB74	*P. fluorescens*	+	+	+	1.3	–	–
AUPB75	*P. fluorescens*	–	+	+	1.3	–	+
AUPB49	*Pseudomonas* sp.[b]	–	+	+	1.2	–	+

Table 3. Continued.

Isolate	Identity (API kit and 16S rDNA analysis)[a]	HCN	Flu.	Sid.	DYZ [cm]	Siderophore type	
						Cat.	Hy.
AUPB04	*P. koreensis* [c]	+	±	+	1.0	–	+
AUPB76	*P. fluorescens*	+	±	+	0.9	–	–
AUPB07	*Pseudomonas sp.*[b]	–	+	+	0.8	–	+
AUPY77	*P. putida*	–	+	+	0.8	–	+
AUPY78	*P. fluorescens*	–	+	+	0.8	–	–
AUPY44	*Pseudomonas* sp.[b]	+	–	+	0.7	–	–
AUPY11	*Pseudomonas* sp.[c]	–	+	+	0.7	–	–
AUPY79	*P. putida*	–	+	+	0.7	–	–
AUPY80	*P. putida*	–	+	+	0.7	—	–
AUPB81	*P. fluorescens*	+	+	+	0.6	–	+
AUPB13	*Pseudomonas* sp.[b]	+	±	+	0.6	–	+
AUPB09	*Pseudomonas* sp.[b]	–	+	+	0.6	–	+
AUPB82	*P. fluorescens*	–	+	+	0.6	–	–
AUPY83	*P. putida*	–	+	+	0.6	–	–
AUPB84	*P. putida*	+	+	+	0.6	+	–
AUPY85	*P. putida*	–	±	+	0.5	–	–
AUPY27	*Pseudomonas* sp.[b]	+	+	+	0.5	+	–
AUPY86	*P. fluorescens*	+	+	+	0.5	+	–
AUPB87	*P. fluorescens*	+	+	+	0.5	+	–
AUPB15	*Pseudomonas* sp.[b]	+	+	+	0.5	+	–
AUPY45	*Pseudomonas* sp.[b]	–	+	+	0.5	–	–
AUPB02	*Pseudomonas* sp.[c]	+	±	+	0.4	–	+
AUPY88	*P. fluorescens*	–	+	+	0.4	–	–
AUPB16	*Pseudomonas* sp.[c]	+	+	+	0.4	+	–
AUPY26	*Pseudomonas* sp.[c]	–	+	+	0.4	–	+
AUPY89	*P. putida*	–	+	+	0.4	–	+
AUPY90	*P. putida*	-	+	+	0.4	–	–
AUPB06	*Pseudomonas* sp.[c]	+	+	+	0.3	+	–
AUPY91	*P. putida*	–	+	+	0.3	–	–
AUPY10	*P. chlororaphis* [c]	+	+	+	0.3	–	+
AUPB40	*Pseudomonas* sp.[b]	–	±	+	0.2	–	+

[a] = Muleta et al. (unpubl.), [b] = PCR-RFLP analysis, [c] = identification supplemented by PCR-RFLP and 16S rDNA analyses, DYZ = diameter of the yellow zone, Flu = fluorescence, Cat = catecholate type, Hy = hydroxamate type, Sid = siderophore, ND = not determined, – = negative for the tested trait, + = positive for the tested trait, ± =extremely equivocal.

Cyanide production, however, was not restricted to the fluorescent pseudomonads as previously noted [35] because non-fluorescent isolates and/or isolates with equivocal fluorescence also produced HCN (see Tab. 3). The color of the picrate/Na_2CO_3-impregnated paper strips changed from yellow (control) to light brown, brown or reddish brown as an indication of the strength of the cyanide produced (data not shown).

3.3 Siderophore Production

One hundred rhizobacterial isolates were qualitatively evaluated for siderophore production. A considerable number of coffee-associated rhizobacteria (67 %) produced siderophores. Almost all were members of the pseudomonads (mainly fluorescent; see Tab. 3). Siderophore production by rhizospheric pseudomonads is well established [15] and the rhizosphere abundance of heterogeneous *Pseudomonas* spp. has been well documented [36]. Their agronomic merits are closely associated with their ability to produce siderophores and other phytobeneficial traits [15].

The yellow zone diameter ranged from 0.2 to 3.9 cm, and 50.7 % of the rhizobacterial isolates tested showed zones greater than 1 cm (see Tab. 3). The largest yellow zone was formed around the colonies of strain AUPB24 (*P. chlororaphis*) after 48 h of incubation. Thirty-five of the 67 *Pseudomonas* spp. that tested positive for siderophores, produced the hydroxamate type of siderophore, but only 12 of the 67 isolates produced the catecholate type (see Tab. 3). Our results are in contrast to those of Joshi et al. [37], who demonstrated that rhizospheric pseudomonads predominantly produced the catecholate type siderophores together with small amounts of the hydroxamate type. The production of various types of siderophores by members of pseudomonads has been verified previously [15].

Among the Enterobacteriaceae, only isolate AUSB14 (*Serratia marcescens*) produced siderophores that were of the catecholate type (see Tab. 3). Enterobacteria typically produce either catecholate or hydroxamate siderophores [38].

In the present investigation, none of the test isolates produced both types of siderophores simultaneously (see Tab. 3). Coincident production of catecholate and hydroxamate siderophores has been reported for some *Pseudomonas* spp. [37], *Agrobacterium* spp. [39] and some members of the Enterobacteriaceae [40].

It should be mentioned here, that yellow haloes on CAS assay plates for siderophore production could also be caused by acidification of the bacteria tested. This could be a reason, why 29 % of the *Pseudomonas* spp. tested positive on the CAS-plates were negative for either of the siderophores. Other reasons could be attributed to the low sensitivity and specificity of the chemical assay methods employed [41], in addition to the detection failure when a compound does not fall into the category of either hydroxamate or catecholate types, since Penyalver et al. [39] claim that some *Agrobacterium* spp. produce a catecholate-hydroxamate type compound similar to the pyoverdines produced by *Pseudomonas* spp., which react in both chemical tests, indicating the low specificity of these assays. The inherent drawback of the current chemical methods means that detection assays for siderophores should be considered mostly being qualitative [41].

3.4 Suitability of the Media for Antagonistic Studies

Bacteria and fungi were cultured on different media to assess a suitable medium which could support the simultaneous growth of both groups (see Tab. 2). All the bacterial isolates and two fungal pathogens (*F. oxysporum* and *F. stilboides*) tested showed good growth on half-strength KB (KBHS) medium (see Tab. 2). However, synthetic low-nutrient medium (SNA) that favored the growth of *Fusarium* spp. did not suit the growth of bacterial antagonists (*Bacillus* or *Pseudomonas* spp.). Similarly, *F. xylarioides* showed very poor growth with the methods employed for *F. oxysporum* and *F. stilboides*, even with an extended period of incubation (15 days), either with or without bacterial cultures (data not shown). Relatively better growth was observed when fungal suspensions and bacterial agar blocks were used on KBHS (Fig. 1G). The in vitro antagonistic studies, therefore, were carried out using KBHS as a standard medium for simultaneous growth of the competitors.

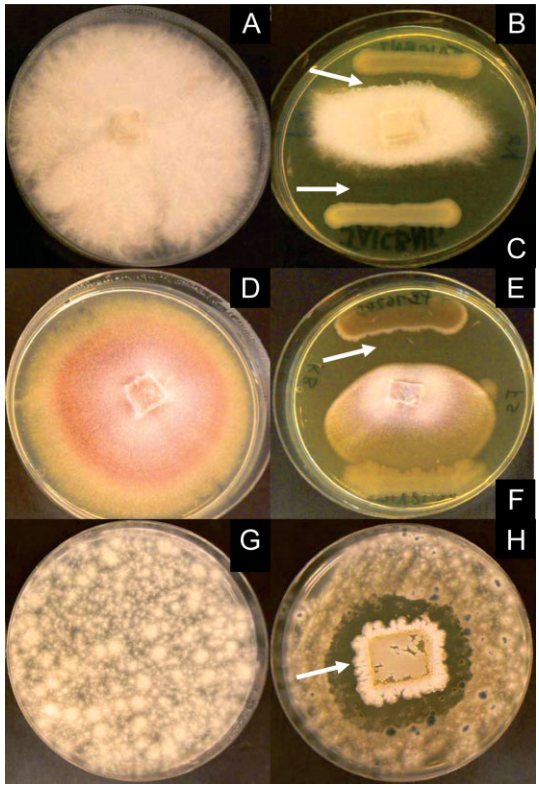

Figure 1. Control plates (left row) and dual culture media showing some rhizobacteria and coffee pathogen interactions: (A) *F. oxysporum*, (B) *P. chlororaphis* (AUPB23) vs. *F. oxysporum*, (C) *P. chlororaphis* (AUPB24) vs. *F. oxysporum*, (D) *F. stilboides*, (E) *Pseudomonas* sp. (AUPB15) vs. *F. stilboides*, (F) *Bacillus* sp. (AUBY95) vs. *F. stilboides* (no inhibition), (G) *F. xylarioides*, (H) *B. subtilis* vs. *F. xylarioides*. Arrows indicate the zones of inhibition.

Although cumbersome, the dual culture medium method still plays a pivotal role in plant pathogenic biocontrol studies [42].

3.5 Screening for Antagonism

Of the 395 available rhizobacterial isolates, a total of 212 isolates were randomly selected and screened for their potential to inhibit the targeted fungal coffee pathogens in in vitro assays. The antagonistic strains tested showed varying levels of effects against the fungal coffee pathogens. Over 10 % of the rhizobacterial isolates showed potent antifungal activity by completely restricting mycelial expansion towards the spotted areas on dual culture media during screening (see Figs.1B, C, E and H). The majority did not show any antagonistic effects (see Fig. 1F). A total of 13 of 83 Gram-negative isolates (all *Pseudomonas* spp.) and 10 of 129 Gram-positive isolates (mainly *Bacillus subtilis*) were quite active antagonistic bacteria against the *Fusarium* spp. (see Tab. 4). The ability to inhibit pathogenic fungal growth is widespread among rhizobacteria, e.g. *Bacillus* and *Pseudomonas* spp. [43]. However, until now, no antagonistic effects of either fungal or bacterial strains have been reported against the deleterious coffee pathogenic *F. xylarioides* and *F. stilboides*, although there are many reports on *F. oxysporum* being inhibited by e.g. *Bacillus* spp. [44].

There were significant differences (F = 6.5 to 8.4, $p < 0.01$) between rhizobacterial antagonists in inhibiting the mycelial expansion of *F. oxysporum* and *F. stilboides,* ranging from 40.1–71.8 % and 37.3–73.6 % radial growth inhibition, respectively (see Tab. 4). Over 82 % of the rhizobacterial antagonists tested (n = 23) exhibited remarkable fungal radial growth inhibition, with a significant percentage (>56 %) against *F. stilboides*, whereas only 30.4 % showed the same degree of effect against *F. oxysporum* (see Tab. 4). Many of the efficient rhizobacterial isolates also showed clearly discernible inhibition of *F. xylarioides* by the complete absence of fungal mycelium in the inhibition zone surrounding a bacterial colony (see Fig. 1H). The rhizobacterial antagonists showed highly significant variations (F = 35.6, $p < 0.01$) in restricting the fungal mycelial growth. Twenty-one of the 23 bacterial isolates exhibited remarkable inhibition against mycelial spread of *F. xylarioides* on dual culture medium by producing large inhibition zones that exceeded 3.0 cm (see Tab. 4). Isolates AUBB20 (*B. subtilis*), AUPB24 (*P. chlororaphis*), AUPB50 (*Pseudomonas* sp.), AUBB12 (*B. subtilis*) and AUBB05 (*B. subtilis*) were the most aggressive inhibitors (see Tab. 4). Antagonists against *F. xylarioides* are highly important because this species causes more severe attacks and ultimately significant yield losses of coffee than any other *Fusarium* species [6].

Overall, isolate AUPB24 (*P. chlororaphis*) was the most efficient inhibitor against all the fungal pathogens tested (mainly *F. stilboides* and *F. oxysporum*), whereas isolate AUBB20 (*B. subtilis*) had the best effect against *F. xylarioides.* Furthermore, isolates AUPB15 (*Pseudomonas* sp.) and AUBB05 (*B. subtilis*) exerted their maximum inhibitory effect against *F. stilboides* (see Tab. 4). Fluorescent pseudomonads (*P. chlororaphis* and *P. fluorescens)* [45]) and *Bacillus* spp. [44] have been reported to be the most frequent beneficial and antifungal rhizobacteria.

For visual observation of clear inhibition zones, the dual medium used was more suitable for Gram-positive isolates (see Fig. 1H) compared with Gram-negative antagonists. Production of diffusible pigments by *Pseudomonas* spp. probably causes the difficulty in observing clear zones.

Table 4. Percentage inhibition of the radial growth/inhibition zone diameter of fungal coffee pathogens due to coffee-associated rhizobacterial antagonists.

Isolate	Identity (PCR-RFLP and 16S rDNA analyses)[a]	Fungal radial growth inhibition [%][b]		Inhibition zone [cm][b]
		F.s	F.o	F.x
AUPB24	*P. chlororaphis*	73.6±1.2a	71.8±1.3a	4.8±0.0ab
AUPB15	*Pseudomonas* sp.	71.8±5.9a	55.5±5.7abcde	3.4±0.4fghij
AUBB05	*B. subtilis*	71.7±2.6a	49.0±3.5cde	4.4±1.8abc
AUBB37	*B. subtilis*	69.3±3.7a	50.7±0.9bcde	3.6±0.7efghi
AUPB02	*Pseudomonas* sp.	69.3±0.6a	56.3±3.1abcde	3.5±0.1fghi
AUPY10	*P. chlororaphis*	68.8±2.2a	57.9±7.4abcde	3.9±0.1cdef
AUBB52	*B. subtilis*	65.6±1.6ab	41.1±2.6e	3.3±1.2ghij
AUPB21	*P. chlororaphis*	65.6±5.8ab	67.9±0.5abc	3.5±0.3fghi
AUBB19	*B. subtilis*	64.0±5.0ab	48.1±3.3de	3.8±1.1cdefgh
AUPB06	*Pseudomonas* sp.	62.0±8.6ab	50.9±3.2bcde	3.1±0.6ij
AUPB16	*Pseudomonas* sp.	61.0±7.2ab	51.6±4.5bcde	3.6±1.9efghi
AUPB23	*P. chlororaphis*	61.0±2.8ab	69.1±1.9ab	4.2±0.8bcde
AUBB20	*B. subtilis*	59.3±9.2abc	61.5±2.3abcd	5.0±2.1a
AUBY48	*Bacillus* sp.	59.2±3.4abc	49.7±3.7cde	3.1±1.3ij
AUBB12	*B. subtilis*	59.1±8.8abc	50.3±10.0bcde	4.3±2.4bcd
AUBY53	*B. subtilis*	57.8±3.0abc	46.1±1.1de	3.9±1.6cdef
AUBB38	*B. cereus*	57.2±1.5abc	49.0±4.8cde	2.8±1.7jk
AUPB07	*Pseudomonas* sp.	57.1±1.1abc	43.5±5.7de	2.4±0.5k
AUPB22	*P. chlororaphis*	56.4±3.6abc	60.2±1.5abcd	3.2±0.6hij
AUBB35	*Bacillus cereus*	47.4±3.6bcd	49.0±11.5cde	3.1±1.0ij
AUPB01	*P. chlororaphis*	46.8±0.8bcd	54.1±3.5bcde	3.7±0.4defghi
AUPB04	*P. koreensis*	41.6±2.2cd	40.1±2.8e	3.1±0.9ij
AUPB50	*Pseudomonas* sp.	37.3±5.6c	54.3±1.2bcde	4.7±0.2ab

F.s = *F. stilboides*, F.o = *F. oxysporum*, F.x = *F. xylarioides*, [a] = Muleta *et al.* (unpubl.), [b] = Data are means ± SD from two replications and values followed by different letter(s) indicate significant differences ($p < 0.05$).

3.6 Possible Mechanisms of Inhibition by HCN-Production

Plant beneficial soil microorganisms exert an array of mechanisms to inhibit mycelial expansion of target fungal pathogens [14, 16, 17, 19]. In the current investigation, all the *Pseudomonas* spp. that showed remarkable inhibitory effects against the test fungal pathogens produced HCN, with the exception of isolate AUPB07 (*Pseudomonas* sp.) (see Tab. 3). This compound, although reported as a potential inhibitor of enzymes involved in major plant metabolic processes [25], is currently attracting remarkable attention and wide applications in areas of biocontrol methods. In addition, not all cyanogenic rhizobacteria are plant growth inhibitory [35], e.g. *Pseudomonas* spp. have been implicated in suppression of soil-borne fungal diseases [17], weed seedlings [33] and plant parasitic nematodes [46].

3.7 Possible Mechanisms of Inhibition by Siderophore Production

The authors' findings revealed that all the potent antagonistic *Pseudomonas* spp. produced siderophores, mainly of the hydroxamate type (see Tab. 3). Loper & Henkels [15] have demonstrated that siderophores produced in situ by *Pseudomonas* spp. chelate iron in a form that is unavailable to pathogens, thereby preventing pathogen access to the already limited pool of soluble iron in the rhizosphere. These authors further remarked that plant growth-promoting rhizobacteria produce siderophores with higher Fe^{+3} affinity than the siderophores produced by deleterious rhizosphere microorganisms, which in turn leads to iron unavailability for the latter microorganisms.

The authors found a strongly positive correlation ($r = 0.70$, $p < 0.05$) between the yellow zone diameter on CAS medium and fungal growth inhibition for *F. oxysporum* but not for *F. stilboides* or *F. xylarioides* ($p > 0.05$), which could be attributed to small yellow zones being produced by some isolates although they showed potent inhibitory activity against target fungal pathogens (see Tabs. 3 and 4). It has previously been observed that slow growing cultures make smaller haloes on siderophore agar plates than fast growing cultures [26].

Apart from the strong antifungal activity and the improving plant iron uptake, siderophores have also been implicated in inducing systemic resistance (ISR) in plants [47]. The implication of siderophores in ISR is highly important in withstanding the deleterious effect of *F. xylarioides* because varietal resistance is one of the most effective methods for controlling this emerging coffee wilt disease [48].

3.8 Possible Mechanisms of Inhibition by Hydrolytic Enzyme Production

In this study, all the bacterial antagonists produced hydrolytic enzymes (lipase and protease) under in vitro conditions (see Tab. 5). However, only seven (all *B. subtilis*) demonstrated β-1,3-glucanase activity, while two isolates (*Bacillus* sp. and *P. koreensis*) showed chitinase activity (see Tab. 5). Evidence is increasing that pseudomonads and bacilli exhibit antifungal activity by producing lytic enzymes [16, 49] in addition to other inhibitory biomolecules. The rhizobacterial isolates (this study) that were positive for chitinase activity had significant radial growth inhibition (> 40 %) against *F. oxysporum* and *F. stilboides* and caused remarkably large inhibition zones (> 3 cm) against *F. xylarioides* (see Tabs. 4 and 5). Nevertheless, the majority of the potent antagonists in our hands showed no in vitro chitinase activity, which suggests the presence of other more common inhibition mechanisms.

Potent and novel antifungal biomolecules are emerging from time to time as more research groups become involved in such areas. Very recently, Kai et al. [19] demonstrated that small organic volatile compounds emitted from bacterial antagonists strongly inhibit mycelial growth of soilborne phytopathogenic fungi.

Table 5. Production of some lytic enzymes by coffee-associated rhizobacterial antagonists.

Isolate	Identity[a]	Production of antifungal agents			
		Lip	Pro	β-1,3-Glu	Chi
AUPB24	*P. chlororaphis*	+	+	–	–
AUPB15	*Pseudomonas* sp.	+	+	–	–
AUBB05	*B. subtilis*	+	+	+	–
AUBB37	*B. subtilis*	+	+	+	–
AUPB02	*Pseudomonas* sp.	+	+	–	–
AUPY10	*P. chlororaphis*	+	+	–	–
AUBB52	*B. subtilis*	+	+	+	—
AUPB21	*P. chlororaphis*	+	+	–	–
AUBB19	*B. subtilis*	+	+	+	–
AUPB06	*Pseudomonas* sp.	+	+	–	–
AUPB16	*Pseudomonas* sp.	+	+	–	–
AUPB23	*P. chlororaphis*	+	+	–	–
AUBB20	*B. subtilis*	+	+	+	–
AUBY48	*Bacillus* sp.	+	+	–	+
AUBB12	*B. subtilis*	+	+	+	–
AUBY53	*Bacillus* sp.	+	+	+	–
AUBB38	*B. subtilis*	+	+	–	–
AUPB07	*Pseudomonas* sp.	+	+	–	–
AUPB22	*P. chlororaphis*	+	+	–	–
AUBB35	*Bacillus* sp.	+	+	–	–
AUPB01	*P. chlororaphis*	+	+	–	–
AUPB04	*P. koreensis*	+	+	–	+
AUPB50	*Pseudomonas* sp.	+	+	–	–

[a] = Isolate identity and order as in Tab. 4, Lip = lipase, Pro = protease, β-1,3-Glu = β-1,3-glucanase, Chi = chitinase.

On the other hand, our in vitro results indicate that the production of antibiotics or antifungal biomolecules by the antagonists, in addition to extracellular enzymes, could also be responsible for fungal growth inhibition. In all the effective antagonists ($n = 23$), a direct contact between fungal mycelia and bacterial colonies on dual culture medium was not observed (see Figs. 1B, C, E and H), suggesting the inevitable diffusion of inhibitory substances, possibly antibiotics along with lytic enzymes into the growth medium. It has previously been indicated that antibiosis is the general mode of antagonism observed for *Bacillus* [50] and *Pseudomonas* spp. [14].

As a complement to these present studies on the antagonistic effects of rhizobacteria against fungal coffee pathogens, the authors also quantified the arbuscular mycorrhizal fungi (AMF) spore density in the coffee rhizosphere in Bonga natural coffee forest, southwestern Ethiopia [3]. In addition, coffee-associated rhizobacteria were assessed for insoluble phosphate solubilization capacity, phytohormone production and utilization of 1-amino-cyclopropane-1-carboxylate (ACC) as a sole nitrogen source, the findings of which will be reported elsewhere (Muleta et al. unpubl.). Many of these coffee-associated microbes showed production of phytohormones, phosphate solubilization, enhancing the P-uptake via AMF, and degradation of ACC, traits that are in multifaceted ways useful to the plant [13, 51–54].

All of the *Pseudomonas* and two *Bacillus* antagonists (this study) solubilized P and of these, three produced indoleacetic acid and two utilized ACC as their sole nitrogen source (*Pseudomonas* spp). The authors also found a considerable degree of AMF root colonization of coffee seedlings from which the rhizobacteria were originally isolated in an earlier work of the authors (Muleta et al., unpubl.). It was suggested that these multiples of beneficial traits and association of AMF with coffee strongly assist the efficiency of candidate antagonists for desired biocontrol methods, emphasizing the great value of concerted mechanisms of action. It has been strongly suggested that the main success of biocontrol agents is largely attributable to their multifunctional characteristics [13] and synergistic interactions with AMF [54], which are of great importance for mycorrhizal dependent Arabica coffee [55].

4 Conclusions

Several coffee-associated rhizobacterial isolates exhibited potent inhibitory effects against deleterious coffee wilt diseases caused by *Fusarium* spp. The antagonists showed more prominent inhibitory activity against *F. xylarioides* and *F. stilboides* than against *F. oxysporum*. Isolate AUPB24 (*P. chlororaphis*) showed the highest percentage inhibition against all the target fungal pathogens.

Only few antagonists showed chitinase activity. The production of siderophores and HCN by *Pseudomonas* spp., lipase and protease by all antagonists and β-1,3-glucanase by several *Bacillus* spp. could be considered the major mechanisms involved in the inhibition of fungal growth. The in vitro findings indicate the potential application of these indigenous rhizobacteria as biocontrol agents due to their efficient inhibitory traits against severe fungal coffee pathogens. Further greenhouse and field trials could ascertain their future applicability for inoculum development.

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