Agar composition affects *in vitro* screening of biocontrol activity of antagonistic microorganisms

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Agar composition affects *in vitro* screening of biocontrol activity of antagonistic microorganisms

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Agar-based screening assays are the method of choice when evaluating antagonistic potential of bacterial biocontrol-candidates against pathogens. We showed that when using the same medium, but different agar compositions, the activity of a bacterial antagonist against *Agrobacterium* was strongly affected. Consequently, results from *in vitro* screenings should be interpreted cautiously.

In recent years, there has been an increasing interest in biological control of soilborne plant diseases, in part as a response to public concerns about hazards associated with chemical pesticides (Dekeyser et al. 2015; Raaijmakers et al. 2002). Basically, biological control of plant diseases is a non-hazardous strategy that involves the use of an organism (or organisms) to inhibit the pathogen and reduce disease (Jaiswal et al. 2015). The main advantage of biocontrol organisms (BCO) is that they are usually not toxic to non-target organisms and less damaging to the environment than chemical pesticides (Hynes & Boyetchko, 2006). In comparison with fungi, bacteria are generally considered ideal BCO of soilborne pathogens because of their rapid growth, easy handling, and fast colonization of the rhizosphere (Sharma 2009; Whipps 2001). The antagonistic potential of candidate BCO has often been tested in a first screening round under *in vitro* conditions using culture media that reflect the nutritional habitat of the pathogens, followed by efficacy testing in bioassays and under field conditions (Köhl et al. 2011). For bacteria, agar overlay assays are generally used to evaluate their antagonistic potential. In this assay melted agar is inoculated with the target pathogen and poured over the surface of cooled solid agar spot-inoculated with the candidate BCO. Following incubation, this results in the selection of those candidates that release a clear area where pathogen growth is inhibited because of its sensitivity to the toxic compounds produced by the BCO (McGarvey et al. 2012). However, as environmental

and nutritional factors may affect microbial growth and metabolite metabolism, the medium composition of *in vitro* screenings may have a significant influence on the screening of microorganisms for commercial use in biological control (Köhl et al. 2011; Tanaka et al. 2014). Therefore, the impact of 11 different agars (Table 1) on the antagonistic interactions between two bacteria was evaluated in this study using the agar overlay assay. Two pathogenic *Agrobacterium* biovar 1 strains (strains NCPPB2659 and ST15.13/097) and one of its antagonistic isolates (strain ST15.15/036; phylum Firmicutes) were used as model organisms. Rhizogenic *Agrobacterium* biovar 1 strains are the causative agent of hairy root disease, representing a major economic problem in hydroponics greenhouses, especially in hydroponics cultivation of tomato and cucumber (Bosmans et al. 2015; Chandra 2012; Weller et al. 2000).

Antimicrobial screening assays were performed in polystyrene petri dishes (Ø 8.0 cm) (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands) filled with 15 mL growth medium, consisting of 20.0 gL^{-1} of the respective agars (Table 1), 3.0 gL^{-1} tryptic soy broth (TSB; Oxoid, Basingstoke, UK), 5.0 gL^{-1} NaCl, and 1.0 gL^{-1} KH_2PO_4. The plates were spot-inoculated with the BCO strain (15 µL per spot; about 10^4 cells per mL in TSB) and incubated for 2 days at 25 °C. Subsequently, 15 mL melted Luria Bertani (LB) agar (20.0 gL^{-1} Merck agar, 5.0 gL^{-1} Bacto™ Yeast extract, 10.0 gL^{-1} NaCl, and 10 gL^{-1} Bacto™ Tryptone) containing *Agrobacterium* (about 6 x 10^5 cells per mL in TSB) was poured over the surface of the plate and incubated again at 25 °C. After 20 hours of incubation, the diameter of the inhibition visible zones was measured (Tyc et al. 2014). Remarkably, only six out of eleven agars revealed antibacterial activity of the test strain (n=8) (Table 1; Fig. 1). Overall, the diameter of the inhibition zones varied between 12.6 mm and 15.2 mm. No differences were observed between both tested *Agrobacterium* strains (Fig. 1). A non-metric multidimensional scaling

(NMDS) plot showed that the elemental concentrations of phosphorus and the metal ions Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Al^{3+} (Table 1) separated the agars in two groups, with one group corresponding to those agars yielding antagonistic activity and the second one comprising agars on which no antagonistic activity was observed (Fig. 2). Specifically Ca^{2+} regulated the activity: all agars on which antagonistic activity was observed contained Ca^{2+} concentrations exceeding 840 mg/kg, whereas Ca^{2+} concentrations were below the detection limit (500 mg/kg) in the agars on which no activity was observed (Table 1). We hypothesized that calcium is needed by the test organism to produce and/or secrete potential toxins/antibiotics against *Agrobacterium* (Dominguez 2004; Zheng & Mackrill, 2016).

To examine the role of calcium in the antagonistic interactions between the tested bacteria, four additional experiments were performed. In a first experiment, agar plates which showed biocontrol activity (Oxoid technical agar No 3) was washed with water (30 min; 25 °C) and subsequently collected by centrifugation (14000 g; 5 min). The deprived agar had lost its ability to produce a zone of inhibition when used in the overlay agar assay (n=2) . In contrast, when the collected washing water was used in combination with an agar which was negative in the first screening (Oxoid bacteriological agar No 1) clear inhibition zones were obtained (n=2). When Oxoid agar No 3 and Oxoid agar No 1 were mixed in a 1/2, 1/5 or 1/10 ratio, the area of obtained inhibition zones decreased proportionally with the amount of Oxoid agar No 1 added (n=2). The largest zone of growth inhibition was obtained for the 1/2 ratio (13.2 mm), followed by the zones obtained for the 1/5 ratio (11.8 mm) and the 1/10 ratio (9.3 mm), indicating the fact that agar media showing biocontrol activity contained specific elements needed to inhibit the growth of *Agrobacterium*. When EDTA (0.05 M or 0.1 M) was added to Oxoid agar No 1 and Oxoid agar No 3 no zones of inhibition were

obtained (n=2), suggesting an important role for bivalent metal cations such as Ca^{2+} which are known to complex with EDTA (Schwarzenbach 1955). Indeed, when $CaSO_4$ was added to Oxoid bacteriological agar No 1 (at a final concentration of 1000 ppm) a clear zone of inhibition was observed (n=2).

Altogether, our study clearly showed that agar composition may substantially affect the outcome of *in vitro* screenings for candidate BCO (see also Bode et al. 2002). Consequently, when screenings would be performed on only a single test medium, several candidate antagonists may be missed. Production of secondary metabolites such as toxins and antibiotics often depends on specific environmental and nutritional conditions (Raaijmakers et al. 2002; Zheng & Mackrill, 2016). Therefore, it is recommended to perform *in vitro* screenings using different types of agar, or to perform a prescreening first on a subset of test isolates on different agar media before screening huge microbial collections. For the particular antagonistic interactions investigated in this study, our results suggested an important role of Ca^{2+} to produce and/or secrete potential toxins/antibiotics against rhizogenic agrobacteria. Whereas the importance of Ca^{2+} as a cell regulator is well established in eukaryotes (Bode et al. 2002), little is known about the precise role of Ca^{2+} in prokaryotes. Nevertheless, recent research suggests the possibility that, as in eukaryotes, Ca^{2+} plays a role in signal transduction in bacteria modulating specific functions or generating a specific respons (Dominguez 2004). The exact function of Ca^{2+} in the antagonistic interaction investigated here remains, however, to be unravelled.

Despite the advantages of a screening under *in vitro* conditions using agar plates, several studies have shown that there may be no correlation between antagonism assessed under *in vitro* conditions and *in planta* (Knudsen et al. 1997; Köhl et al. 2011).

Therefore, further research is needed to develop novel (preferably general) reliable *in vitro* selection media, that reflect the typical nutritional habitat of the microorganisms, and systems for high-throughput screening of potential bacterial biocontrol agents. Preferably, such efficacy testing under *in vitro* conditions should not bias preferring single mode of actions such as toxin production, but should rather allow the detection of candidates with promising combinations of different modes of action or even still unknown modes of action (Köhl et al. 2011).

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Conflict of Interest

No conflict of interest declared.

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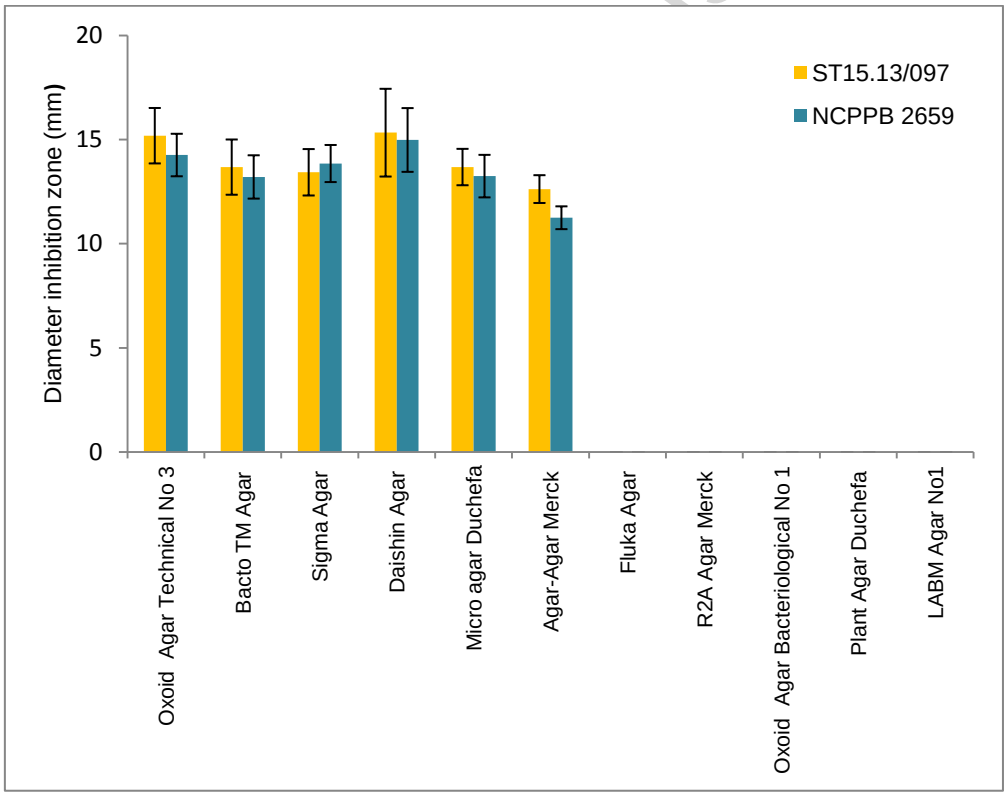
Figure

Figure 1. Antagonistic activity of isolate ST15.15/036 against rhizogenic *Agrobacterium* biovar 1 (strains NCPPB2659 and ST15.13/097) using an overlay assay with different agars. (A) Mean inhibition zone (n = 8). Error bars represent standard errors. (B) Illustration of medium showing antagonistic (left) or no antagonistic activity (right).

A



B

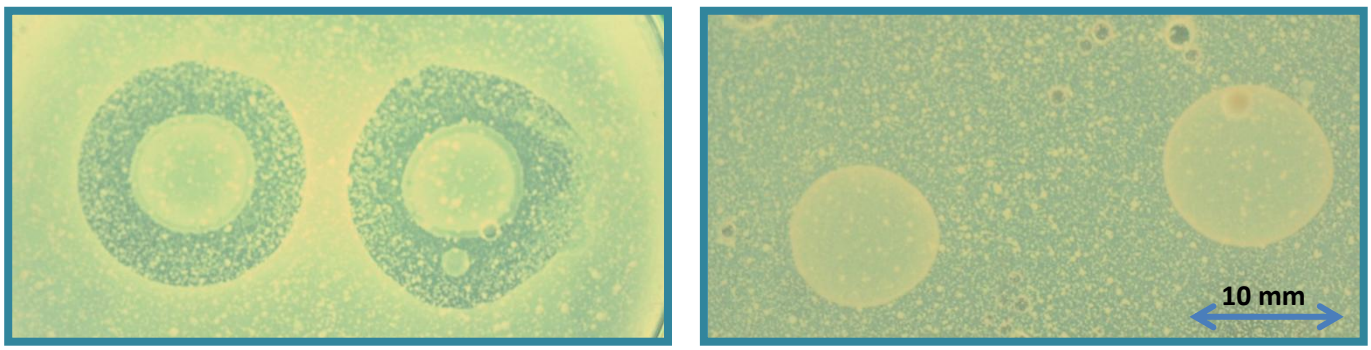
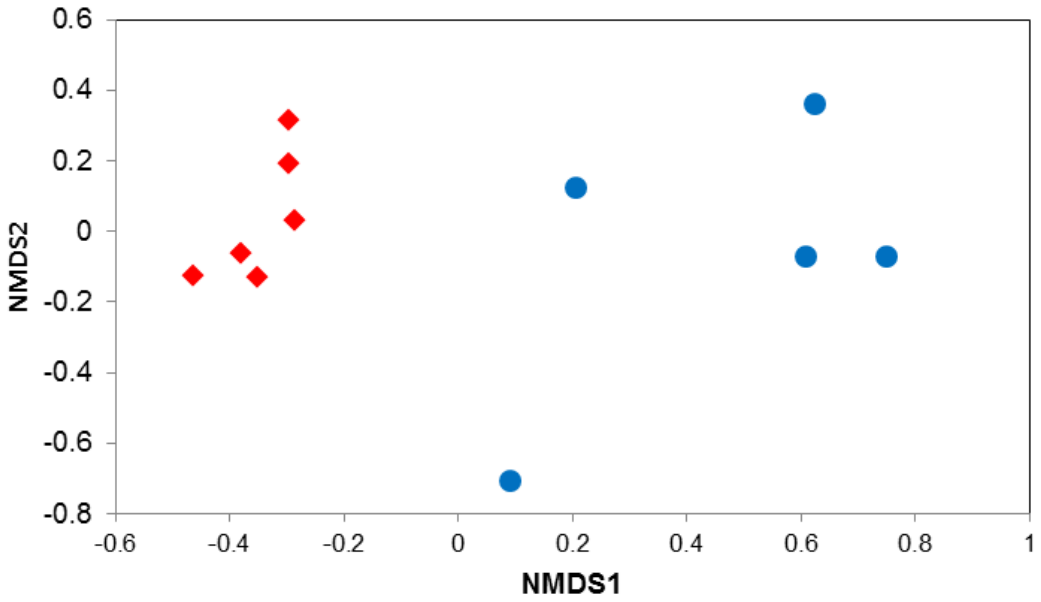


Figure 2. Non-metric multidimensional scaling (NMDS) ordination of the chemical composition (see Table 1; values below the detection limit were replaced by "0" (zero)) of the agars studied (stress value = 0.0605). Red diamonds and blue circles represent agars showing antagonistic and no antagonistic activity of isolate ST15.15/036 against rhizogenic *Agrobacterium* biovar 1 (strains NCPPB2659 and ST15.13/097), respectively.



Tables

Table 1. Agars used in this study[a].

Agar	Provider	Growth inhibition[b]	Metal concentrations(mg/kg)									
			Cu^{2+}	Zn^{2+}	CaO	P_2O_5	K_2O	MgO	Na_2O	Fe^{2+}	Mn^{2+}	Al^{3+}
Oxoid LP0013 Agar Technical No 3	Oxoid	+	<[c]10	<[c]20	3300	<[c]500	<[c]500	1140	9000	17.2	3.14	8.46
Bacto TM Agar BD 214010	Bacto TM	+	<10	<20	2300	<500	<500	820	8500	30.7	1.57	9.52
Sigma Agar A1296-500 Agar-Agar	Sigma Aldrich	+	<10	<20	1660	<500	<500	1110	7400	32.3	2.09	31.3
Daishin Agar Brunschwig Chemie	Brunschwig chemie	+	<10	<20	920	<500	<500	970	2000	124	20.90	80.4
Duchefa micro agar	Duchefa Biochemie	+	<10	<20	2600	<500	<500	1260	8900	21.7	7.83	11.6
Agar-Agar Merck 1.01614.1000	Merck	+	<10	<20	840	610	<500	<[c]500	15800	24.2	2.54	76.6
Fluka Agar 05038-500	Sigma Aldrich	-	<10	<20	<[c]500	<500	<500	<500	3900	14.9	<[c]0.50	9.03
R2A Agar Merck 1.00416.0500	Merck	-	<10	<20	<500	9400	11900	610	23000	26.8	20.70	1.05
Oxoid LP0011 Agar Bacteriological No 1	Oxoid	-	<10	<20	<500	4500	<500	<500	12100	14.6	<0.50	8.93
Plant Agar Duchefa Biochemie P1001.1000	Duchefa Biochemie	-	<10	<20	<500	<500	<500	650	2400	32.4	19.40	29.8
LABM Agar No1 Bacteriological MC002	LAB M	-	<10	<20	<500	8400	560	<500	18900	34.5	<0.50	16.1

[a] For each agar metal ion concentrations were determined using ICP-OES as described by Olesik (1991). Concentrations are expressed in mg per kg of dry matter.

[b] Bacterial antagonism of isolate ST15.15/036 against rhizogenic *Agrobacterium* biovar 1 (strains NCPPB2659 and ST15.13/097) was evaluated using the overlay agar assay: +, inhibition zone observed; -, no inhibition zone.

[c] <LOD, below the detection limit.