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Hygienic characteristics of radishes grown in soil contaminated with *Stenotrophomonas maltophilia*

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Abstract

Background: *Stenotrophomonas maltophilia* is a plant growth-promoter. This bacterium is also implicated in human diseases. Thus, after the use of this bacterium in agriculture, the safety of the final products has to be verified. Due to the ubiquitous presence of *S. maltophilia* in soil, in this study a massive contamination was simulated to evaluate the growth and safety of *Raphanus sativus* L..

Results: Different inoculums and soil treatment conditions were tested. Soils were analysed weekly and the radishes at harvest for their microbial loads and presence/persistence of *S. maltophilia* LMG 6606. The concentration of the bacterium added in the different trials decreased during the first week, but increased thereafter and determined a significant increase of growth parameters of radishes.

Conclusions: The addition of *S. maltophilia* LMG 6606 to non-autoclaved soil enhanced the productivity of radishes. The bacterium did not internalize in the hypocotyls, but colonized the external surface ensuring the safety of the products. Thus, a sanitizing bath of hypocotyls before consumption is necessary.

Keywords: Hygienic safety, Microbial internalization, Plant growth, *Raphanus sativus* L., *Stenotrophomonas maltophilia*

Background

Soil fertility is a complex concept that involves many interacting parameters. Cultivated plants may suffer nutritional stresses when the amount or availability of soil nutrients is lower than that required for sustaining metabolic processes in each growth stage [1]. Thus, restoring of nutrients and enhancing their availability by improving soil characteristics and efficiency of plants, are the main objectives of the modern agriculture. Due to the increasing sensitivity to environmental and economic issues, researchers and consumers are more and more aware of the impact of agriculture on the environment. Lowering the use of chemical inputs and the search for alternative ways to improve a more sustainable agriculture is a current challenge [2].

Sustainable agricultural production systems may be obtained by enhancing the uptake efficiency of nutrient of plants, that might be achieved through a better comprehension of the role of plant–microbe–soil interaction and the association of soil microorganisms with roots in the rhizosphere [3]. Within soil microbiota, plant growth-promoting (PGP) microorganisms constitute a heterogeneous group of bacteria and fungi that have gained particular importance for their stimulating effects. They are able to promote directly plant metabolism by nutritional and/or hormonal ways [4–9] as well as indirectly through the production of antimicrobial compounds, the reduction of iron available to phytopathogens, the synthesis of fungal cell wall-lysing enzymes, the competition with detrimental microorganisms for colonization sites on the roots, and the induced systemic resistance [5, 10].

The most studied PGP rhizobacteria belong to Gram-negative genera [11]. Among these, *Stenotrophomonas maltophilia* is an important species and represents one of the four dominant bacterial species in the rhizosphere of European cereal cultivations [12]. *S. maltophilia* is a Gammaproteobacterium distributed worldwide [13] and

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typically found in soil where it plays a defining role in the nitrogen and sulphur cycles [14–16] and it often dominates the microbial communities detected outside and inside the plants [13]. However, *S. maltophilia* is a known human pathogen [17]. It may be responsible for respiratory tract infections, bacteremia, biliary sepsis, infections of the bones and joints, urinary tract and soft tissues, endophthalmitis, eye infections, endocarditis and meningitis [18].

In the last years, the change in lifestyle and the consequent need of consuming ready-to-eat foods determined the increase of fresh-cut vegetable use. Since these products do not undergo any treatment before consumption, their hygienic safety is of paramount importance. Among fresh vegetables, the request of radish is on the increase [19] due to its positive effect on the consumer's health [20–22].

Radish (*Raphanus sativus* L.) is an important vegetable of the *Brassicaceae* family, grown and consumed all over the world, due to its wide adaptation, high yield, and high nutritional content. It is normally consumed in salads and comes in a variety of forms and skin colors. Most known varieties are round and red-skinned. The most popular part for eating is the napiform hypocotyl, although the entire plant is edible and the tops can be used as a leaf vegetable. Radishes are eaten raw or cooked or processed by pickling, canning or drying [23]. Radish is not only a vegetable crop but also an important source of medicinal compounds. In fact, it is used by people with different gastrointestinal, biliary, hepatic, urinary and respiratory disorders, and in cardiovascular diseases such as hypertension [24]. An important quality characteristic of radish is the total antioxidant activity due to molecules, such as ascorbic acid and phenol, mainly phenolic acids, with free radical scavenging activity [25, 26]. The protective role of these molecules in the prevention of human degenerative diseases has been widely demonstrated by many studies [27–29]. Moreover, as other Crucifers, radish contains many other compounds as glucosinolates that are associated with cancer protection [30].

The interaction between *S. maltophilia* and *R. sativus* has been recently approached in a floating cultivation system [31]. However, their reciprocal effects in soil and the safety aspects related to the safety of the radishes have not been evaluated yet. In the present study high levels of *S. maltophilia* were added to soil, simulating a massive environmental contamination, to: investigate the survival of *S. maltophilia* in soil during the whole crop cycle of *R. sativus*; evaluate the effects of this bacterium on plants; monitor its transfer to the radishes; and determine their viability in the radishes ready for consumption.

Methods

Microbial strain, plant seeds and experimental plan

Stenotrophomonas maltophilia LMG 6606, a strain originating from rhyzosphere as reported in the strain details provided by the Belgian Co-ordinated Collection of Micro-organisms (BCCM/LMG), was propagated in Nutrient Broth (NB) (Oxoid, Milan, Italy) at 28 °C for 24 h. Seeds of radish (*R. sativus* L.) cultivar Saxa three were purchased from Blumen (Piacenza, Italy).

The experimental plan included eight different conditions for the growth of radish plants: ASS, autoclaved soil inoculated with *S. maltophilia* LMG 6606; AS, autoclaved soil added with Ringer's solution (Sigma-Aldrich, Milan, Italy); nASS, non-autoclaved soil inoculated with *S. maltophilia* LMG 6606; nAS, non-autoclaved soil added with Ringer's solution; ASSwS, autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606; ASwR, autoclaved soil weekly added with Ringer's solution; nASSwS, non-autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606; nASwR, non-autoclaved soil weekly added with Ringer's solution. Four replicate pots were produced for each trial. Two independent experiments were performed in two consecutive weeks.

Inoculation of soil and plant development

The pots used in this work were 13.5 cm × 13.5 cm × 16 cm and were filled with 2.5 L of commercial soil SER CA-V7 (Vigorplant Italia srl, Piacenza, Italy). This soil is a mixture of slightly or fully decomposed raised bog peat (pH 6.0) fertilized with 800 g m^{-3} of a mineral fertilizer (NPK 12-11-18). The pots were previously treated with a NaClO solution (5 % *v*/*v*) for 24 h. A part of the bulk soil, placed in autoclave bags, was autoclaved twice (in two consecutive days) for 70 min at 120 ℃ [32]. The fresh inoculums of *S. maltophilia* LMG 6606 was prepared after overnight development as reported by Settanni et al. [33]. The cell suspension was added to the autoclaved and non-autoclaved soil in the ratio 1:10 (*v*/*v*), vigorously mixed with sterile spoons to obtain the homogenous distribution of the bacterial inocula and transferred into the pots (trials ASS, nASS, ASSwS and nASSwS). The trials AS, nAS, ASwR and nASwR were prepared with Ringer's solution. Seeds of radish were sown in five dibblings for each pot. Pots were watered from below (sub-irrigation) with sterile water, in order to avoid further microbial contamination to the soil and kept at 25 ℃ in a climatic chamber till seed germination. From the third day, the trials ASSwS and nASSwS were weekly added with 300 mL of *S. maltophilia* LMG 6606 cell suspension (concentration at about 10^8 CFU mL^{-1}), while the trials ASwR and nASwR were added with the same volume of Ringer's solution.

After seed emergence, only five plants per pot were left to grow (274 plants m^{-2}). The pots were transferred in an unheated plastic greenhouse and received the same volume of water that varied daily according to environmental conditions and plant needs until harvesting.

Microbiological analyses

The sampling for microbiological analysis included soil during plant growth, aseptically collected as described by Settanni et al. [33] at T_0 and at 7-day intervals, and hypocotyls at harvest, aseptically collected as reported by Settanni et al. [31]: four hypocotyls were collected from each replicate of all trials, two hypocotyls for the direct microbial count and two for the internal *S. maltophilia* LMG 6606 detection.

Soil samples (10 g) were diluted (1:10) with sodium pyrophosphate (0.16 % *w*/*v*) solution in sterile flasks under agitation (10 min at 150 rpm). Radishes (approximately 10 g) were first subjected to the removal of the soil adhering to the surface as described by Brandl et al. [34] and then homogenised in Ringer's solution by a stomacher (BagMixer® 400, Interscience, Saint Nom, France) at the maximum speed for 2 min. The decimal serial dilutions of both soil and radishes continued in Ringer's solution. Total mesophilic count (TMC) were determined on Plate Count Agar (PCA) (Oxoid), incubated aerobically at 30 ℃ for 72 h; presumptive *S. maltophilia* were enumerated on vancomycin–imipenem–amphotericin B (VIA) agar [35], incubated aerobically at 30 ℃ for 48 h. Plate counts were performed in duplicate.

Data from bacterial counts were averaged and converted to log CFU g^{-1} dry weight (dw) for soil samples and to log CFU g^{-1} for radishes. Moisture of soil (5 g) was obtained after drying (24 h at 105 ± 1 ℃) in an oven and weighting the residual.

Recognition of *S. maltophilia* LMG 6606 and evaluation of the internalization

Approximately ten colonies from the two highest dilutions of sample suspensions at each analysis were picked up from VIA agar plates based on their morphology (colour, edge, surface and elevation) and cultured in NB overnight at 30 °C. The cultures were sub-cultivated onto NA and stored in glycerol (20 %, *v*/*v*) stocks at −80 °C. The isolates and *S. maltophilia* LMG 6606 were analysed by randomly amplified polymorphic DNA-PCR (RAPD-PCR) as described by Settanni et al. [31].

The internal presence of *S. maltophilia* LMG 6606 in radishes was investigated as reported by Settanni et al. [31]. Briefly, radishes of each trials were collected, superficially sterilized and then transferred in stomacher bags, added with VIA broth (final ratio 1:10) and homogenized as reported above.

Analyses of plants

Plants were harvested 4 weeks after sowing and washed accurately with tap water. After air drying, leaves, hypocotyls and roots were separated and the number of leaves and their area, the root elongation and the radish diameter were recorded on four plants for each replicate of each trial. Leaf area of each plant was calculated by digital image analysis. Leaves were scanned (Epson Perfection 4180 Photo, Seiko Epson Corp. Japan) with 350 dpi of resolution and the images were saved in TIFF format. The images were analysed with the ImageJ 1.46r software (National Institutes Health, Bethesda, MD, USA).

Fresh and dry biomass of the different plant parts were calculated by weighting before and after they were oven dried to a constant weight at 80 ℃.

Color of radishes was measured on two points of four hypocotyls from each replicates of all trials, using a colorimeter (Chroma Meter CR-400C, Minolta, Osaka, Japan). The Hunter scale parameters were determined: *L* (lightness, ranging from 0 to 100, from black to white), *a* (positive values indicating redness and negative values, greenness) and *b* (positive values indicate yellowness and negative values, blueness).

The firmness of radishes was determined using a digital penetrometer (mod. 53205, TR Snc. Italy) equipped with a flat 6 mm diameter stainless steel cylinder probe. Four hypocotyls were punched and the mean peak force was calculated in Newton.

Radishes (50 g) were homogenized in 50 mL of distilled water and homogenate centrifuged (5000 rpm, 15 min); the supernatant was taken for analysis of soluble solids content (SSC), ascorbic acid and $N–NO_3^-$. SSC was measured using a digital refractometer (MTD-045nD, Three-In-One Enterprises Co. Ltd. Taiwan). Ascorbic acid and nitrate content were measured by the Reflectoquant test strips and a RQflex hand-held reflectometer (Merck, Darmstadt, Germany) [procedures described in Art. 1.16971.0001 and 1.16981.0001 by Merck (http://www.merckmillipore.com/chemicals)].

Water use efficiency (WUE) was calculated as the ratio between total dry weight of plant and total amount of water supplied.

Statistical analyses

The study was carried out in a completely randomized design. To determine the effects of microorganisms and time on soil microbial load, a two-way ANOVA was carried out. A one way ANOVA was performed for other data. When a significant F value was detected, Tukey–Kramer's multiple range test was used to determine differences among microbial populations and plant parameters of the different trials (significance level $P < 0.05$).

Principal components analysis was employed to investigate any underlying relationship among the different trials based on the agronomic and quality parameters of radish plants at harvest. The input matrix for the analysis consisted of leaf number, leaf fresh weight, leaf dry matter, leaf area, root length, root fresh weight, root dry matter, radish fresh weight, radish dry matter, radish diameter, firmness, L^*, a^*, b^*, SSC, nitrate, ascorbic acid, plant WUE. For the selection of the optimum number of principal components (PCs), factors with eigenvalues greater than 1.0 were retained. In addition the plot of the PCs enabled the investigation of correlations between the variables of the input data set. To this end, the initial variables were projected into the subspace defined by the reduced number of PCs (first and second components) and correlated variables were identified. In the current approach, the Principal components analysis was implemented with SPSS version 14.0 (SPSS Inc. Chicago, IL, USA).

Results

Microbiological analyses

The microbiological counts of soil during the growth of radishes are reported in Table 1. The interaction microorganisms x time resulted significant for both PCA and VIA media ($P < 0.001$). The autoclaved soil uninoculated showed a TMC of 3.53 Log CFU gdw^{-1} and, surprisingly, the bacterial load estimated on VIA was at almost 2 Log CFU gdw^{-1}, whereas the non-autoclaved un-inoculated soil showed levels of ca. 7 Log CFU gdw^{-1} on both PCA and VIA media. Soon after inoculation, the concentration of TMC and *S. maltophilia* LMG 6606 or stenotrophomonads were above 10^8 CFU gdw^{-1}. In general, TMC was at higher levels than the microbial developments detected on VIA, but the trials nASS, nASSwS, nAS and nASwR showed an opposite behaviour at 7 day. During radish growth, the highest microbial concentrations were observed for the trial AS and ASwR, whose TMC, at the second week, were 9.58 and 9.57 Log CFU gdw^{-1}, respectively. Lower concentrations were estimated for the trials nASS, nASSwS, nAS and nASwR, for which TMC was below 8 Log CFU gdw^{-1} for the entire period of observation. The concentrations on VIA evaluated for the trials weekly added with *S. maltophilia* LMG 6606 (ASSwS and nASSwS) showed trends almost comparable to those displayed by the corresponding trials not subjected to the weekly additions (ASS and nASS), but the levels estimated for the second were slightly lower.

Table 1 Microbial counts (Log CFU gdw^{-1}) in soil as function of time and trials

Trials[A]	Days				
	0	7	14	21	28
PCA[B]					
AS	3.53 ± 0.37i	7.61 ± 0.66eh	9.58 ± 0.24a	9.16 ± 0.11ab	8.98 ± 0.28ac
ASS	8.21 ± 0.25cf	8.06 ± 0.39dg	8.96 ± 0.30ac	9.03 ± 0.07ac	9.10 ± 0.37ab
ASSwS	8.21 ± 0.25cf	9.11 ± 0.15ab	8.74 ± 0.20ad	9.20 ± 0.31ab	8.85 ± 0.34ad
ASwR	3.53 ± 0.37i	7.29 ± 0.60gh	9.57 ± 0.21a	9.17 ± 0.11ab	9.06 ± 0.22ab
nAS	6.88 ± 0.20h	7.24 ± 0.08gh	7.11 ± 0.15h	7.41 ± 0.35fh	7.65 ± 0.15eh
nASS	8.45 ± 0.34be	7.26 ± 0.10gh	7.52 ± 0.08fh	7.35 ± 0.17gh	7.52 ± 0.18fh
nASSwS	8.45 ± 0.34be	7.30 ± 0.11gh	7.50 ± 0.15fh	7.48 ± 0.08fh	7.57 ± 0.13fh
nASwR	6.88 ± 0.20h	7.04 ± 0.02h	7.09 ± 0.09h	7.25 ± 0.13gh	7.34 ± 0.18gh
VIA[B]					
AS	2.12 ± 0.10p	7.77 ± 0.09ci	8.67 ± 0.04ac	6.91 ± 0.18io	6.61 ± 0.7lo
ASS	8.28 ± 0.02ae	7.19 ± 0.48gm	8.15 ± 0.25af	7.67 ± 0.15dk	8.01 ± 0.44ag
ASSwS	8.28 ± 0.20ae	7.72 ± 0.21ci	8.81 ± 0.08ab	7.18 ± 0.15gn	7.88 ± 0.32bh
ASwR	2.12 ± 0.35p	7.03 ± 0.56ho	8.85 ± 0.30a	7.25 ± 0.29fl	6.71 ± 0.14ko
nAS	6.85 ± 0.10io	7.17 ± 0.08go	6.90 ± 0.04io	6.23 ± 0.26no	6.90 ± 0.36io
nASS	8.35 ± 0.02ad	7.76 ± 0.24ci	7.49 ± 0.23dl	6.22 ± 0.29o	6.68 ± 0.09lo
nASSwS	8.35 ± 0.20ad	7.49 ± 0.11dl	7.35 ± 0.42el	6.97 ± 0.27ho	7.05 ± 0.12ho
nASwR	6.85 ± 0.35io	7.47 ± 0.11dl	7.07 ± 0.32go	6.60 ± 0.36lo	6.27 ± 0.08mo

Data represent the mean of four replicates of two independent experiments. For each media, data followed by the same letter are not significantly different according to Tukey–Kramer's multiple range test at $P < 0.05$

[A] Trials: *ASS* autoclaved soil inoculated with *S. maltophilia* LMG 6606, *AS* autoclaved soil, *nASS* non-autoclaved soil inoculated with *S. maltophilia* LMG 6606, *nAS* non-autoclaved soil, *ASSwS* autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606, *ASwR* autoclaved soil weekly added with Ringer's solution, *nASSwS* non-autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606, *nASwR* non-autoclaved soil weekly added with Ringer's solution

[B] Media: *PCA* plate count agar, *VIA* vancomycin–imipenem–amphotericin B agar

The trials not inoculated with *S. maltophilia* LMG 6606 (AS and nAS) showed levels of count on VIA particularly high. At harvest, the hypocotyls were analysed for TMC and stenotrophomonad concentrations and no statistical significant differences were registered for the microbial concentrations of the radishes cultivated in the eight different conditions (results not shown). TMC were in the range 7.07–7.63 Log CFU g^{-1}, while the counts detected on VIA were at least 1 Log cycle lower for each trial followed.

Monitoring of *S. maltophilia* LMG 6606

All ten presumptive stenotrophomonad isolates collected from VIA medium at the highest dilutions of soil samples were characterized at strain level by RAPD-PCR analysis. DNA from the pure culture of *S. maltophilia* LMG 6606 was used for strain recognition. The comparison of the polymorphic profiles is shown in Fig. 1. *S. maltophilia* LMG 6606 dominated the microbial community found on VIA medium for the trials ASS, nASS, ASSwS and nASSwS until the 28th day of experimentation. Despite the high counts detected on VIA for the trials AS, nAS, ASwR and nASwR, no colony showed a RAPD profile superimposable to that of *S. maltophilia* LMG 6606 excluding, at least at the highest dilutions of soil samples, a cross-contamination among inoculated and un-inoculated trials. One main RAPD pattern was recognised for the stenotrophomonad isolates from the un-inoculated autoclaved soil (AS and ASwR) trials and another main profile for the isolates from the un-inoculated non-autoclaved soil (nAS and nASwR) trials.

The same procedure was applied on the stenotrophomonad isolates collected from VIA medium at the highest dilutions of radishes and it produced the same results (not shown) registered for soil: *S. maltophilia* LMG 6606 dominated the microbial community of radishes for the trials ASS, nASS, ASSwS and nASSwS, whereas no colonies collected from the trials AS, nAS, ASwR and nASwR shared the same *S. maltophilia* LMG 6606 RAPD profile.

The last data could not indicate whether the bacterium added to soil was adherent to the radish surface or in the inner part. Thus, at harvest, the radishes were also specifically investigated for the internal presence of *S. maltophilia* LMG 6606. The enrichment cultures obtained in VIA broth after incubation of the homogenized surface sterilized hypocotyls were streaked onto the corresponding agar medium. No colonies developed for the trials AS, nASS, nAS, ASwR and nASwR (results not shown) indicating that no stenotrophomonads internalized in these conditions. On the contrary, a development was observed for the trials ASS, ASSwS and nASSwS. These colonies, characterized by the same appearance (colour, morphology, edge, surface and elevation), were randomly collected and subjected to the RAPD analysis as reported above; all cultures shared the same profile of *S. maltophilia* LMG 6606 (results not shown) demonstrating its internalization. Interestingly, the only inoculated trial that did not show the internal presence of *S. maltophilia* LMG 6606 in radishes, at harvest, was nASS. To confirm the last data, the enrichment cultures from nASS were also streaked onto Nutrient Agar. Seventy-nine colonies were isolated and analysed by RAPD-PCR (results not shown), but none of them shared the same profile of *S. maltophilia* LMG 6606.

Plant growth

Seed germination and seedling emergence occurred after 3 days from sowing in non-autoclaved soil and 1 day later in autoclaved soil. Inoculation of soil with *S. maltophilia* LMG 6606 had no effect on seed germination.

During the cultivation period, average maximum and minimum temperatures inside the greenhouse ranged between 29.5 and 18.4 ℃, respectively. Soil temperature did not greatly differ from air temperature (29.2 and 17.8 ℃, respectively).

Plant growth was significantly influenced by soil treatment (Table 2). After 28 days from sowing, some differences were registered for the development of plants. The above-ground part showed only little differences in the number of leaves $plant^{-1}$ among the eight trials, but leaf fresh weight of nASS and nASSwS was higher (14.4 and 17.6 g $plant^{-1}$, respectively) than that found for the other trials (10.5 g $plant^{-1}$ on average). No differences were found in dry matter percentage as function of treatments, while leaf dimensions were influenced by soil autoclaving as well as by inoculation with *S. maltophilia* LMG 6606. The lowest leaf area was recorded for the plants of trials AS and ASwR (141.5 cm^2 on average) that differed significantly from that of the plants of trials ASS, ASSwS, nAS and nASwR (193.3 cm^2 on average). The non-autoclaved

(See figure on next page.)

Fig. 1 Monitoring of *S. maltophilia* LMG 6606 during the growth cycle of radish performed by RAPD-PCR profile comparison. Trials: *ASS* autoclaved soil inoculated with *S. maltophilia* LMG 6606, *ASSwS* autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606, *nASS* non-autoclaved soil inoculated with *S. maltophilia* LMG 6606, *nASSwS* non-autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606, *AS* autoclaved soil, *nAS* non-autoclaved soil, *ASwR* autoclaved soil weekly added with Ringer's solution, *nASwR* non-autoclaved soil weekly added with Ringer's solution. *Lanes*: *M* marker (GeneRuler 100 bp Plus DNA ladder, M·Medical Srl, Milan, Italy); *1*, *S. maltophilia* LMG 6606; *2–11*, colonies randomly collected from the highest dilutions of soil samples on VIA agar from each trial

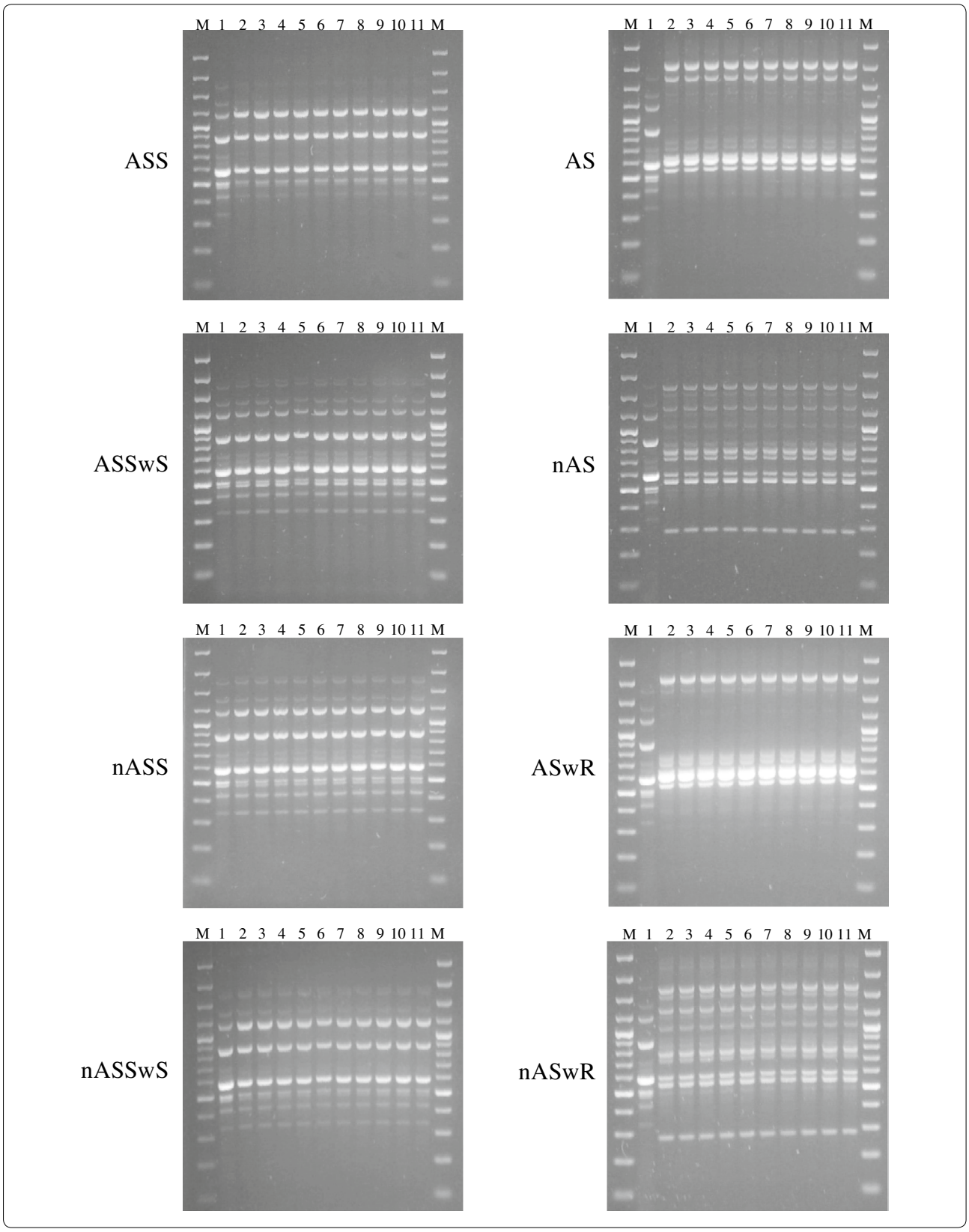
M 1 2 3 4 5 6 7 8 9 10 11 M
ASS
AS
ASSwS
nAS
nASS
ASwR
nASSwS
nASwR

Table 2 Agronomic and quality parameters of radish plants at harvest

Parameters	Trials							
	ASS	ASSwS	AS	ASwR	nASS	nASSwS	nAS	nASwR
Leaves								
Number (n. plant^{-1})	5.0 ± 0.3a	4.8 ± 0.2a	4.8 ± 0.2a	4.7 ± 0.2a	4.6 ± 0.2ab	4.6 ± 0.2ab	4.5 ± 0.3ab	4.1 ± 0.2b
Fresh weight (g)	10.5 ± 0.6c	10.7 ± 0.5c	10.3 ± 0.4c	10.8 ± 0.5c	14.4 ± 0.8b	17.6 ± 0.9a	10.3 ± 0.5c	10.3 ± 0.6c
Dry matter (%)	6.5 ± 0.3a	7.1 ± 0.4a	7.1 ± 0.5a	7.1 ± 0.3a	7.3 ± 0.4a	7.2 ± 0.4a	6.8 ± 0.3a	6.7 ± 0.4a
Leaf area (cm^2)	186.4 ± 8.7b	196.4 ± 9.3b	138.3 ± 6.9c	144.8 ± 7.2c	297.0 ± 16.9a	311.5 ± 14.6a	198.9 ± 9.7b	191.6 ± 9.8b
Roots								
Length (cm)	31.5 ± 1.6ab	33.6 ± 1.8a	28.1 ± 1.4ac	29.1 ± 1.5ac	26.3 ± 1.5bc	23.9 ± 1.1c	27.6 ± 1.4ac	27.8 ± 1.6ac
Fresh weight (g)	1.06 ± 0.05a	1.04 ± 0.04a	0.91 ± 0.05a	0.8 ± 0.04a	0.49 ± 0.02b	0.50 ± 0.03b	0.53 ± 0.04b	0.40 ± 0.03b
Dry matter (%)	6.2 ± 0.3b	6.2 ± 0.4b	7.2 ± 0.5b	7.3 ± 0.4b	7.7 ± 0.4b	8.1 ± 0.5b	12.8 ± 0.6a	11.7 ± 0.5a
Radish								
Fresh weight (g)	12.2 ± 0.7b	10.0 ± 0.6b	10.1 ± 0.5b	12.0 ± 0.6b	25.0 ± 1.4a	23.9 ± 1.2a	15.3 ± 0.8b	14.8 ± 0.9b
Dry matter (%)	3.9 ± 0.2a	3.9 ± 0.3a	3.9 ± 0.4a	3.8 ± 0.2a	4.1 ± 0.3a	4.3 ± 0.5a	4.3 ± 0.2a	4.5 ± 0.4a
Diameter (mm)	24.9 ± 1.2b	23.4 ± 1.2b	23.1 ± 1.3b	24.6 ± 1.2b	34.0 ± 1.7a	31.8 ± 1.6a	25.5 ± 1.3b	26.6 ± 1.5b
Firmness (N)	39.1 ± 2.1a	38.6 ± .0a	36.2 ± 1.8a	37.1 ± 1.9a	30.3 ± 1.7b	29.9 ± 1.5b	31.2 ± 1.6b	30.2 ± 1.5b
*L**	32.9 ± 1.7c	34.0 ± 1.6c	35.2 ± 1.8ac	34.2 ± 1.7bc	36.8 ± 1.9ac	38.7 ± 1.8ab	39.6 ± 2.0a	39.0 ± 2.0a
*a**	45.8 ± 2.3ab	46.0 ± 2.4ab	42.5 ± 2.1b	43.5 ± 2.2b	47.9 ± 2.6a	50.3 ± 2.5a	49.4 ± 2.9a	49.4 ± 2.5a
*b**	20.6 ± 1.0ab	20.7 ± 1.2ab	19.2 ± 1.0b	18.8 ± 0.9b	21.0 ± 1.1ab	23.2 ± 1.2ab	25.1 ± 1.3a	25.0 ± 1.4a
SSC (Brix)	2.2 ± 0.1b	2.2 ± 0.1b	2.8 ± 0.1a	2.6 ± 0.2ab	2.3 ± 0.1b	2.3 ± 0.2b	2.3 ± 0.1b	2.3 ± 0.1b
N–NO_3^- (mg kg^{-1} FW)	644.6 ± 33.2a	580.3 ± 29.8a	583.7 ± 29.2a	563.7 ± 28.2a	522.7 ± 25.1a	543.4 ± 27.8a	567.0 ± 28.4a	599.2 ± 30.2a
Ascorbic acid (mg kg^{-1} FW)	205.0 ± 10.3ab	197.0 ± 9.9ab	220.0 ± 11.0a	206.0 ± 10.3ab	182.0 ± 9.1ab	166.0 ± 8.3ab	132.0 ± 6.6b	192.0 ± 9.6ab
Plant WUE (g DM kg^{-1} H_2O)	2.03 ± 0.11b	2.03 ± 0.10b	2.0 ± 0.11b	2.14 ± 0.12b	3.51 ± 0.18a	3.88 ± 0.16a	2.39 ± 0.12b	2.33 ± 0.13b

Data within a row followed by the same letter are not significantly different according to Tukey–Kramer's multiple range test at $P < 0.05$

Percentages were subjected to angular transformation prior to perform statistical analysis

soils inoculated with *S. maltophilia* LMG 6606, subjected or not to the weekly additions, determined the greatest total leaf area which was about 300 cm^2.

The roots of radish plants had a lower fresh weight in the trials with non-autoclaved soil than autoclaved soil trials, but dry matter content was higher only for nAS and nASwR. Root elongation was influenced by inoculation with *S. maltophilia* LMG 6606. However, this inoculums determined a decrease of root length in non-autoclaved soil trials, while an opposite behaviour was observed for the autoclaved soil trials.

After 28 day from sowing, radish hypocotyls reached commercial maturity. The highest average fresh weight was recorded in nASS and nASSwS (25.0 and 23.9 g, respectively), and was almost twice those reached by the hypocotyls of the other trials. A positive correlation was found between radish fresh weight and size. The biggest hypocotyls developed in non-autoclaved inoculated soils which overcame the diameter of 30 mm. No significant difference was found for dry matter percentage among the eight trials.

Colour modifications of radish were evaluated in terms of L^*, a^* and b^* values. Radishes grown in non-autoclaved soil had a darker colour especially for ASS and ASSwS trials ($L^* = 33.5$ on average), while trials nAS and nASwR determined the greatest values of L^* (39.6 and 39.0, respectively). The inoculation with *S. maltophilia* LMG 6606 did not influence neither the redness (a^*) nor the yellowness (b^*) of radish colour. The trials AS and ASwR were characterised by the lowest redness and differed significantly from non autoclaved trials. The only significant differences for yellowness were noticed between radishes from trials AS and ASwR (19.0 on average) and trials nAS and nASwR (25.0 on average).

Soil autoclaving determined an increase of radish firmness both in inoculated and un-inoculated trials (37.7 N on average), while a lower value was registered for those grown in non-autoclaved soils (30.4 N on average).

Soluble solid contents were almost comparable among the different trials, with the exception of AS that was significantly higher. Also nitrate and ascorbic acid content showed similar values among trials with no significant differences between inoculated and un-inoculated trials.

Stenotrophomonas maltophilia LMG 6606 increased significantly WUE for producing dry matter (DM) when inoculated in non-autoclaved soil trials: nASSwS and nASS ranged from 3.51 to 3.88 g DM kg^{-1} H_2O, respectively, against 2.15 g DM kg^{-1} H_2O on average for the other trials.

Multivariate data analysis

The results of the principal components analysis showed four principal components (PCs) with eigenvalues higher than 1.00 (Table 3), accounting for 56.5, 15.4, 7.7 and 6.34 % of the total variance, respectively. This indicated that the initial 18 variables could be expressed as a linear combination of four PCs explaining 85.9 % of the total variance. PC1 was mainly related to leaf fresh weight, leaf dry matter, leaf area, root length, root fresh weight, root dry matter, radish fresh weight, radish dry matter, diameter, firmness, ascorbic acid, plant WUE and color components (L^*, a^*, b^*); PC2 was related to leaf number and b^*; PC3 was related to SSC, and finally PC4 to nitrate content (Table 3). The projection of the original variables on the plane of the two first PCs could clearly illustrate such relationship as shown in the plot of loadings (Fig. 2a). The discrimination of the various trials can be visualized in the plot of scores (Fig. 2b) where three clusters could be clearly distinguished. The trials with autoclaved soil were close each other and located mainly in the negative part of *F1* axis; they were clearly separated from the trials with autoclaved soil that were located in the positive part of *F1* axis. Among these trials nASSwS and nASwR were clearly separated from nAS and nASS. The trial nASS also showed the greatest score for Factor 1.

Combining the information from the plot of loadings and scores, it can be inferred that nAS and especially nASS influenced positively fresh and dry matter of leaves and radishes, colour and firmness of hypocotyls and plant water use efficiency.

Table 3 Correlation of variables to the factors of the PCA analysis based on factor loadings

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Leaf number	−0.236	*0.716*	0.242	−0.210
Leaf fresh weight	*0.809*	0.427	0.037	−0.065
Leaf dry matter	*0.781*	0.422	−0.070	0.082
Leaf area	*0.870*	0.310	0.072	0.182
Root length	*−0.773*	0.174	0.210	0.457
Root fresh weight	*−0.772*	0.452	0.206	0.017
Root dry matter	*−0.836*	−0.115	0.259	−0.137
Radish fresh weight	*0.940*	0.173	−0.075	−0.006
Radish dry matter	*0.956*	0.107	−0.063	−0.050
Radish diameter	*−0.841*	0.430	0.087	0.080
Firmness	*0.858*	0.242	−0.002	0.073
L^*	*0.794*	−0.518	0.088	−0.128
a^*	*0.771*	−0.398	0.328	0.027
b^*	0.506	*−0.726*	0.397	0.004
SSC	−0.282	−0.072	*−0.842*	−0.338
Nitrate	−0.020	−0.229	−0.392	*0.817*
Ascorbic acid	*−0.721*	−0.411	−0.079	−0.110
Plant WUE	*0.939*	0.283	−0.066	0.011

Values in italic within the same factor indicate the variable with the largest correlation

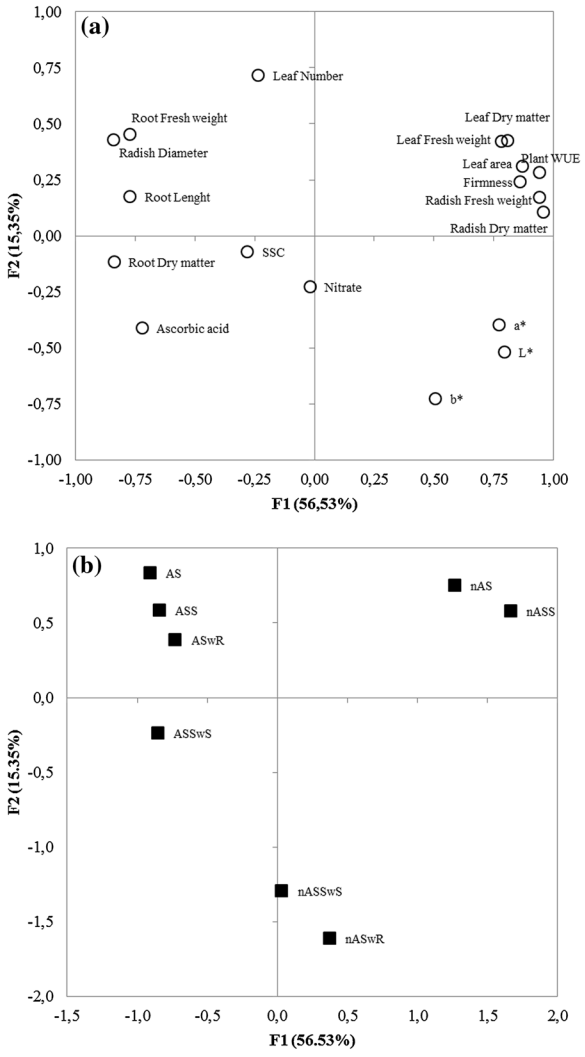

Fig. 2 Plot of **a** loadings (agronomic and quality parameters of radish plants at harvest) and **b** scores (trials) formed by the first two principal components from the PCA analysis. *ASS* autoclaved soil inoculated with *S. maltophilia* LMG 6606, *AS* autoclaved soil, *nASS* non-autoclaved soil inoculated with *S. maltophilia* LMG 6606, *nAS* non-autoclaved soil, *ASSwS* autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606, *ASwR* autoclaved soil weekly added with Ringer's solution, *nASSwS* non-autoclaved soil weekly inoculated with *S. maltophilia* LMG 6606, *nASwR* non-autoclaved soil weekly added with Ringer's solution

Discussion

The positive effects of *S. maltophilia* on the plant growth has been reported for several species [36–39]. However, *S. maltophilia* is also reported to be a human pathogen. For this reason, its association with vegetables, especially when eaten raw, is detrimental for the final quality of these products. The aim of this work was to modify the microbial composition of soil with the addition of *S. maltophilia* LMG 6606 in order to simulate a massive contamination and to assess its effects on the growth of *R. sativus*.

The experimentation was carried out with soil amended with inorganic fertilizer; organic amendments were not included to avoid the transfer of consistent concentrations of microorganisms [33] commonly present in compost or manure. The trials were carried out in a greenhouse during the spring season, with an average air temperature of 24.0 and 23.5 ℃ reached in the soil. In this conditions, *S. maltophilia* LMG 6606 found a temperature range compatible with its growth.

Stenotrophomonas maltophilia may increase the plant growth not only by direct production of growth promoting substances but also indirectly by interacting with the native root microflora [40]. Kwok et al. [41] reported that the biocontrol ability of *S. maltophilia* might be enhanced in presence/combination with other soil microorganisms. For these reasons, our experimental design included autoclaved and non-autoclaved soils, in order to exclude or include, respectively, the interaction between *S. maltophilia* LMG 6606 and the resident soil microbiota. Plate counts specific for the added bacterium were performed on VIA medium that, to our knowledge, is still considered to be highly selective for the isolation of *S. maltophilia* [35]. The non-autoclaved un-inoculated soils showed an initial load of 6.92 Log CFU gdw^{-1} on VIA. Thus, for the trials carried out with non-autoclaved un-inoculated soil (trials nAS and nASwR), these populations were referred to as stenotrophomonads. This bacterial group was detected also in the soil soon after autoclaving, although at very low levels (2.13 CFU gdw^{-1}). Thus, the common protocol applied to sterilize soil (two autoclaving cycles at 121 ℃ for 70 min at 24 h interval) was not enough, in this study, to destroy completely the bacterial component and, as a consequence, the microbial interaction could not be totally excluded in the autoclaved soil trials, at T_0.

The trials artificially contaminated with more than 10^8 CFU gdw^{-1} of *S. maltophilia* LMG 6606 showed a decrease of these inocula during the first week of radish growth, but their number increased thereafter. De Boer et al. [42] registered an opposite trend during the first week of observation of sands inoculated with stenotrophomonads, but the stimulating effect was imputed to the development of fungi. However, the different behaviour of this bacterial population might be due to the different conditions tested in the two works; the soil amended with inorganic fertilizer used in our work and the sand used by De Boer et al. [42] represented two distinct ecosystems, characterized by differences in pH, water activity and oxidation–reduction potential. Furthermore, the differences can also depend on the strain-specific characteristics. A continuous decrease of *S.*

maltophilia inoculated at 10^7 CFU gdw^{-1} in different soil types with different management regimes at 28 ℃ was reported by Messiha et al. [43] who studied the antagonistic effects of *S. maltophilia* against *Ralstonia solanacearum*. Those authors stated that the reasons for the differences in survival of *S. maltophilia* in the various soils were not clear, but supposed a direct effect of nitrate and ammonium contents and pH. The increase in concentration of *S. maltophilia* LMG 6606, observed in this study from the second week, might be due to the production of methionine by *R. sativus* roots. *S. maltophilia* requires methionine [44] and colonizes mainly the rhizosphere of cruciferous plants which produce high concentrations of sulphur-containing compounds [45].

The persistence of *S. maltophilia* LMG 6606 was monitored by plate count and polymorphic profile by RAPD analysis, after isolation. The isolates collected from VIA agar at the highest dilutions of soils were analyzed and compared with the pure strain. The direct comparison of RAPD patterns allowed to confirm that *S. maltophilia* LMG 6606 dominated the stenotrophomonad group of soil in all the inoculated trials during the entire growth cycle of radishes. This approach was previously successfully applied on the recognition and monitoring of other food/spoilage bacteria tested in similar experiments [31, 33].

In our study, the strain *S. maltophilia* LMG 6606 determined a significant increase of the values of several parameters (leaf area, fresh and dry matter yield, radish size and WUE) indicating the growth-promotion of radish, even though the plants grown in presence of *S. maltophilia* LMG 6606 behaved differently in autoclaved or non-autoclaved soil. Plants exhibited a significantly greater growth in the latter condition. This might be explained with the fact that soil treatments, especially steaming or autoclaving, can result in soil toxicity [46].

Dry matter yield of control trials was similar to those obtained by other authors [47]. The increase in yield was significant only for non-autoclaved soil and ranged from 47 to 67 % for nASS and nASSwS, respectively. The growth increase may vary greatly depending on crop and PGP rhizobacteria strains used [48]. Antoun et al. [47] reported an increase of 15 % in the dry matter yield of radish using the strain *Bradyrhizobium japonicum* Tal 629.

The results of the principal components analysis showed four principal components (PCs). Combining the information from the plot of loadings and scores, it can be concluded that soil autoclaving negatively influenced growth and quality parameters, thus their use need further investigation. Furthermore, principal components analysis was able to differentiate the trials with respect to agronomic and quality trait of radish. Hence, soil autoclaving should be avoided, while the inoculation of non-autoclaved soil with *S. maltophilia* LMG 6606 only once before plant establishment was positively related with some growth parameter.

Radishes were microbiologically investigated at harvest. TMC and stenotrophomonad concentrations were not statistically different for all trials. Thus, inoculated trials produced radishes characterized by the same levels of microbial contamination of those obtained from control trials. The levels of TMC were in the range 10^6–10^7 CFU g^{-1} and were superimposable to those reported for radishes grown in hydroponic systems [31].

The isolates collected from the highest dilutions of hypocotyls confirmed that *S. maltophilia* LMG 6606 dominated the microbial community of radishes for all inoculated trials as assessed by RAPD analysis. However, to retrieve the exact location of this bacterium on the radishes, inside or outside the hypocotyls, they were surface sterilized and subjected to an enrichment in VIA broth. This procedure, developed specifically for radishes in a previous work [31], indicated that *S. maltophilia* LMG 6606 internalized in all inoculated trials except that carried out in non-autoclaved soil not subjected to the weekly addition (nASS).

Conclusions

The addition of *S. maltophilia* LMG 6606 to the non-autoclaved soil amended with inorganic fertilizer enhanced the productivity of radishes grown in greenhouse. *S. maltophilia* LMG 6606 colonized the external surface of the hypocotyls, but it did not internalize. Thus, a massive contamination of soil with *S. maltophilia* determines a growth promotion of *R. sativus*, but due to the hygienic implication of this bacterium, a sanitizing bath of hypocotyls before eating is mandatory. Works are being prepared to better evaluate the biocontrol activity of *S. maltophilia* in presence of other soil microorganisms in specific combinations.

Authors' contributions

AMi provided financial support of the experiment and conducted greenhouse experiment and agronomic and quality determinations, general conception and coordination of the experiments, interpretation of results and manuscript writing. AMa carried out the microbiological and molecular genetic analyses. GM provided general conception and interpretation of results and manuscript writing. LS provided financial support of the experiment, offered expert advice during the conduction of the assays, interpretation of results and manuscript writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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