Title: **Suitability of mycorrhiza-defective mutant/wildtype plant pairs (Solanum lycopersicum L. cv Micro-Tom) to address questions in mycorrhizal soil ecology**

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4 **Suitability of mycorrhiza-defective mutant/ wildtype plant pairs (*Solanum*

5 ***lycopersicum L.* cv Micro-Tom) to address questions in mycorrhizal soil ecology**

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Abstract

Despite the importance of arbuscular mycorrhizal fungi (AMF) to ecosystem processes, few experimental tools are available to quantify AMF contributions to process rates. In this study we examine the efficacy of an experimental system consisting of wildtype (WT) and different non-mycorrhizal (Myc-) genotype pairs of tomato (*Solanum lycopersicum L.*), specifically focusing on cv Micro-Tom. Two conditions necessary to make such a system useful were examined; A) that the Myc- genotype(s) do not get colonized in a full soil AMF community background, while the WT does, and B) that there are no non-target effects of the Myc- phenotype on soil microbes. We assessed the second condition by growing Myc- genotypes and WT in non-mycorrhizal soil, monitoring plant growth (root, shoot biomass; root length; root diameter size distribution) and soil microbial community structure (PLFA analysis) as indicators of any changes in root tissue quality or rhizodeposition. All tested Myc- genotypes showed a drastically reduced colonization in mycorrhizal soil. However, in non-mycorrhizal soil, M161 had greater root biomass and M20 greater microbial biomass compared to WT. Only one of the Myc- mutants examined fully met the criteria. We conclude that the BC1/ WT pair is a powerful experimental system and recommend caution when using Myc- mutants in mycorrhizal ecology.

Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with the majority of land plants (Smith and Read 1997). Despite the pervasive influence this symbiosis can have on a variety of terrestrial ecosystem processes (Allen 1991; Rillig 2004), gaining experimental access to the quantitative contribution of AMF to process rates has proven a challenge, since few methods exist to single out AMF effects (Rillig 2004). No method exists to specifically eliminate AMF from soil, and researchers have resorted to using general fungicides, such as benomyl, to approximate this effect (e.g. Smith et al. 2000; Hartnett and Wilson 2002; Callaway et al. 2004). Alternatively, soil biota can be eliminated and AMF selectively added back to the experimental system. While a valid and often-used experimental practice, disturbance introduced by methods aimed at eliminating soil biota (e.g., steaming, chloroform-fumigating, autoclaving) could introduce a number of confounding effects (Endlweber and Scheu 2006). Intricate associations between AMF and other soil biota (e.g., Rillig et al. 2006) in particular, cannot be easily re-established using microbial washes (Koide and Li, 1989) or other methods to reintroduce soil microbes. Application of tracer isotopes can permit measurement of nutrient fluxes, e.g. phosphate (Schweiger and Jakobsen 2000) and recently, rotated core-designs in which a static core (allowing AMF hyphal access) is paired with rotated meshed cores (continuously breaking AMF links) have been successfully used to gain access to effects of the mycorrhizal mycelium (Johnson et al.

2001, 2002). However, the latter method is also not disturbance-free. Therefore, there is still a need to explore other experimental systems that might afford greater experimental control and flexibility.

The recent availability of non-mycorrhizal (Myc-) plant mutants, derived primarily for the biochemical and physiological study of plant-fungus interactions (e.g., Peterson and Guinel 2000; Gadkar et al. 2001; Marsh and Schultze 2001; Harrison 2005; Paszkowski et al. 2006), makes possible a different approach to studying the role of AMF in plant-soil processes. The clear advantage of using Myc- mutants is that the experimental design is non-invasive, since AMF do not have to be eliminated from the system. As a consequence, this approach appears particularly suitable for studies of AMF influences on plant/ soil processes mediated by soil/ rhizosphere microbial communities (Cavagnaro et al. 2007a). Therefore, a number of studies have employed Myc-/ WT (wild type) pairs to address such soil and plant ecological questions (e.g. Augé et al. 2004; Marschner and Timonen 2005; Neumann and George 2005 [in this study the WT used was not the mutant progenitor]; Barker et al. 2006; Cavagnaro et al. 2004, 2006, 2007ab).

Given the increasing popularity of such Myc-/ WT pairs, we here evaluated several new mutant tomato genotypes (Table 1) in regards to their relative suitability for addressing soil microbiological questions. For these genotypes/ pairs data are not yet available that would permit their use in asking soil microbial and ecological questions. Our evaluation criteria for this purpose include:

(1) In the presence of an entire AMF community derived from a complex inoculum source (roots, hyphae and spores) with the full soil microbiota background, the Myc- tomato genotype(s) will not, and the WT genotype will, become colonized by AMF. The non-mycorrhizal habit of Myc- mutants has generally been verified under fairly controlled, artificial conditions with defined inoculum sources representing only a few species of AMF (e.g., Barker et al. 1998; Gao et al. 2001; David-Schwartz et al. 2001, 2003; Sekhara Reddy et al. 2007), but there are also thorough tests under field conditions (Cavagnaro et al., 2006). Much of the work in this regard has been carried out with tomato (*Solanum lycopersicum L.* Mill) *rmc* mutants (reduced mycorrhizal colonization; Barker et al., 1998). The *rmc* mutant restricted colonization by several AM fungi to the formation of appressoria, abortive penetrations of epidermal cells and extraradical hyphae (surface colonization), but permitted extensive cortical root colonization by at least one isolate (Gao et al. 2001). However, the *rmc* mutants did not become colonized in the field or using field soil, where a fungal community (consisting of several species) must have been present (Cavagnaro et al. 2006, 2007ab).

(2) Grown in the absence of AMF, WT and Myc- genotype(s) will exhibit similar growth parameters and will give rise to similar soil microbial communities (an indicator for tissue quality and rhizodeposition). Several researchers have tested these assumptions for other tomato plants. Cavagnaro et al. (2004) and Poulsen et al. (2005), for example, found no differences in growth within their plant pairs, but one study did report differences in gross growth parameters between Myc- and WT genotypes (for the tomato pair *rmc*/*76R*: Marschner and Timonen 2005). While unusual in the literature, this latter

result calls into question the uncritical use of such pairs. Differences that occur between the genotypes in the absence of AMF must be due to non-target effects of the mutagenesis process, and may therefore confound ecological hypothesis testing.

Materials and Methods

Plant material

Table 1 gives an overview of the tomato plant genotypes used in the experiments described below. The Micro-Tom cultivar was originally selected for mutagenizations since it has small stature and fast generation time, properties that facilitated screening for Myc- mutants (Meissner *et al*, 1997). These properties are also advantageous for a model system for microcosm-based soil ecology studies.

Experiment 1: Mycorrhizal soil

In order to demonstrate that the mutant(s) will not become colonized in a full soil background, we grew them and the WT in a non-sterilized soil. Plants (all genotypes listed in Table 1; n = 5) were grown in 235 mL pots (one plant per pot) filled with field soil from a local grassland (pH = 6.6; organic matter content 5.72%; texture: 63.5% sand, 21.5% silt, 14.5% clay; Kjeldahl N = 0.32%; Olsen-P = 10.8 mg g^{-1}; Lutgen et al. 2003). AMF are abundant at the site (Lutgen et al., 2003), and numerous AMF phylotypes are present (Mummey and Rillig 2006; 2007). As no propagule types were removed, spores,

hyphae and colonized root fragments would have all been available in the soil to contribute to root colonization. All pots were placed in a growth chamber with 18 hours light, 60% relative humidity, temperature at 20 ˚C, and watered as needed (2-3 times per week). After 9 weeks of growth, roots were extracted from the soil and their dry weight determined. Root lengths were measured using the WinRhizo V 3.10B root image analysis system (Régent Instruments Inc, Québec, Canada). AMF root colonization was measured microscopically at a magnification of 200X after staining with Trypan Blue, as described in Rillig et al. (1999). We obtained AMF-colonized root length (in meters) by multiplying percent root colonization by root length. Additionally, soil extraradical hyphal lengths of AMF were measured microscopically at 200X after aqueous filtration-extraction and staining with Trypan Blue (Rillig et al. 1999). We distinguished AMF hyphae from that of other fungi by a set of morphological criteria described previously (Rillig et al. 1999). One plant in each of the WT and BC1 groups died, and samples from these pots were not analyzed.

Experiment 2: Non-mycorrhizal soil

The second condition for successful use of WT/Myc- experimental pairs is that plants perform similarly in the absence of mycorrhizal fungi. In this experiment, we steamed field soil (same as Experiment 1) to eliminate any AMF inoculum. We added other soil microbiota back to the pots using a filtered wash of the non-sterile soil (50 mL per pot); the wash was obtained by passing soil solutions through Whatman No. 1 paper filters

with 11µm particle retention size (this method was sufficient to retain any AMF propagules including hyphae; data not shown). As mentioned above, there are limitations to this re-introduction of soil microbiota, but this is a widely practiced method, and it allowed us to test for effects on (re-established) microbiota for the purposes of this study. Tomato plants (all genotypes listed in Table 1; n = 10) were grown (one plant per 235 mL pot) in a greenhouse under natural day light, supplemented with 14 hours of additional light, for 10 weeks. Plants were watered to field capacity as needed (2-3 times per week). At harvest, roots and shoots (including fruits) were separated and dry weights determined. We also measured root length and diameter size distribution using the WinRhizo V 3.10B root image analysis system. AMF root colonization was also assessed, as above.

As an indicator of any differential effects of roots or their products, we analyzed soil microbial communities on a subset of five randomly selected samples from each treatment. For this purpose, soil (50 mL) from each pot was immediately frozen after mixing. Soil microbial communities were analyzed by phospholipid fatty acid analysis (PLFA). PLFA analysis was chosen since it generally is more sensitive than DNA-based methods (Ramsey et al. 2006), in part because it may also capture physiological responses. PLFA extraction and analysis was carried out as previously described (Ramsey et al. 2005). Briefly, PLFAs were extracted and analyzed according to the method of White and Ringelberg (1998) and analyzed as described by Frostegård et al. (1993). Lipids were removed from 5 g soil into chloroform using a modified Bligh and Dyer extraction procedure. Phospholipids were separated from other lipids by silicic acid

chromatography and derivatized to fatty acid methyl esters (FAMEs) for analysis by gas chromatography. Total PLFA content (ng g^{-1} soil) was used as a measure of microbial biomass according to Frostegård and Bååth (1996).

Statistical analysis

Plant growth and mycorrhiza data were analyzed using analysis of variance (JMP 3.1.6.2, SAS Institute). Normal distribution of residuals was verified using Shapiro-Wilk W test, and homogeneity of variances with Levene's test. Percentage data were arcsine square-root transformed to obtain normality. When assumptions of homogeneity were violated despite transformation, we carried out Welch-ANOVA, which adjusts degrees of freedom to allow for differences in variation among groups, or Kruskall-Wallis tests. Means comparisons were conducted posthoc (only after ANOVA was significant at $P < 0.05$) using Tukey-Kramer HSD for comparisons of data meeting parametric assumptions, or Kruskal-Wallis multiple-comparison Z-value test (NCSS 2001) for data that did not.

Relationships between the PLFA data of all samples were examined using ordination techniques. Initial detrended correspondence analysis indicated that the data exhibited a linear, rather than unimodal, response, justifying the use of linear ordination methods (Lepš and Smilauer 2003). Therefore, relationships between samples were evaluated by redundancy analysis using CANOCO software (Microcomputer Power, Ithaca, NY). This technique finds the portion of the variability that can be explained by experimental treatments. A Monte Carlo permutation test based on 499 random permutations was used

to test the null hypothesis that bacterial community profiles were unrelated to tomato genotype.

Results

Experiment 1: Mycorrhizal soil

While there were no significant differences in root weight (ANOVA; $F_{3, 14} = 1.62$; $P = 0.23$) or root length (ANOVA; $F_{3, 14} = 0.42$; $P = 0.74$), AMF root colonization was significantly different among the tomato genotypes (Welch ANOVA; $F_{3, 7.04} = 9.66$; $P < 0.007$), with substantially higher colonization in the WT than the other groups (20 to 25 times higher in the WT than in M20 or BC1; Table 2). This general pattern also held for arbuscular root colonization (Welch ANOVA [arcsine square-root transformed data]; $F_{3, 6.29} = 15.9$; $P = 0.002$), with M161 intermediate in value. Colonized root length (in meters) differed significantly among plants (Kruskall-Wallis H = 9.01, $P = 0.03$), and was highest for WT, and lowest for BC1 and M20, with M161 intermediate (even though the latter was not significantly different [Z-test] from WT owing to large variability). Extraradical AMF hyphal lengths in the soil followed the same pattern (ANOVA; $F_{3, 14} = 7.8$; $P = 0.003$), with WT plants giving rise to twice the hyphal lengths of any other group (Table 2).

Experiment 2: Non-mycorrhizal soil

None of the roots were found to be colonized by AMF (data not shown). Final plant shoot and root dry weights are shown in Fig. 1. Although shoot dry weight was not significantly different among the different genotypes (ANOVA; $F_{3, 36} = 1.24$; $P = 0.31$), there were differences in root weights (ANOVA; $F_{3, 36} = 8.29$; $P = 0.0003$), with M161 plants having significantly greater root mass than other plants.

Total root length did not differ significantly among the groups ($F_{3, 36} = 0.96$; $P = 0.42$; Fig. 2). There were also no differences detected for average root diameter between groups ($F_{3, 36} = 1.23$; $P = 0.31$; data not shown). Length of five different root diameter size classes was recorded between 0.0 to 1.0 mm. Multivariate analysis of variance indicated that there were marginal differences (Wilks' Lambda = 0.48; $P = 0.050$), justifying the examination of individual response variables. Univariate analyses of variance indicated that two root diameter size classes exhibited significant differences: 0.4-0.6 mm ($F_{3,36} = 4.16$; $P = 0.01$) and 0.8-1.0 mm (Welch-ANOVA; $F_{3, 19.3} = 3.96$; $P = 0.02$), while the other three did not (0.0-0.2 mm: $F_{3, 36} = 0.25$; $P = 0.86$; 0.2-0.4 mm: $F_{3, 36} = 2.58$; $P = 0.07$; 0.6-0.8: $F_{3, 36} = 2.79$; $P = 0.054$). For these two diameter size classes, no significant differences were found between WT and other plants, only among M161 and M20, and M20 and BC1 genotypes, respectively.

Soil microbial biomass (represented by sum total of PLFAs) was significantly different between genotypes (ANOVA; $F_{3,19} = 3.34$; $P = 0.045$; Fig. 3). In particular, M20 had an about 50% higher mean microbial biomass than WT (planned pairwise comparison; $P =$

0.006). None of the other genotypes differed from WT (planned pairwise comparisons; $P > 0.20$).

Analysis of microbial community structure, as indicated by redundancy analysis of PLFA mole-percentages, showed no significant differences between tomato genotypes ($F = 0.93$; $P = 0.51$; Fig. 4). Further examination of individual PLFAs with t-tests revealed only one significant difference between WT and BC1 samples at $P < 0.05$ (cy17:0); an effect that disappeared with adjustment for the large number of comparisons which were made.

Discussion

Our aim was to critically test a set of novel tomato Myc-/WT pairs for their suitability in soil microbial ecology studies by checking behavior in mycorrhizal and non-mycorrhizal soils. The most important finding was that not all mutant genotypes tested here were suitable, and that therefore careful scrutiny of new mutants (or continued testing of this and more established mutants) is an important task.

Behavior in mycorrhizal soil

Results from Experiment 1 demonstrated that all mutants were less colonized than the WT in a non-treated field soil. The WT was highly colonized with both hyphae and arbuscules compared to the mutants, with production of extraradical hyphae providing additional evidence of a functional mycorrhiza. Our results show that only the M20 and

BC1 genotypes exhibited strongly reduced colonization with hyphae or arbuscules in a full field soil background. Our results confirm that M161 is less suppressive than M20 (David-Schwartz et al. 2003); this was particularly evident for the colonized root length data. Previous work demonstrated that M20 and M161 can resist colonization only under certain controlled conditions with specific AMF isolates (David-Schwartz et al. 2001, 2003; Table 1). Hence, our results are an important extension of these findings for the purpose of developing MicroTom WT/ Myc- pairs for plant-soil interaction studies. While all propagules in the field soil were included, hyphae eminating from neighboring plants were not; thus our results should be critically re-examined for plant-plant interaction studies.

All Myc- genotypes are tested by exposing them to AMF; however, often this occurs under controlled conditions with frequently only few species of AMF as inoculum (e.g., Barker et al. 1998; Gao et al. 2001; David-Schwartz et al. 2001, 2003; Poulsen et al. 2005; Sekhara Reddy et al. 2007). The tomato *rmc*/ WT pair is probably the best-studied example for which colonization tests under field conditions or using field soil have occurred (e.g., Cavagnaro et al. 2006, 2007ab). These are important for two reasons: (a) AMF may differ in their ability to colonize mutant roots (Gao et al. 2001); and (b) in a field microbial background microbes that facilitate colonization could be present, for example mycorrhization helper bacteria (Garbaye 1994; Xavier and Germida 2003; Frey-Klett et al. 2007).

Behavior in non-mycorrhizal soil

The second criterion is far more difficult to test, since it is essentially impossible to completely rule out every non-target effect on the plant's physiology. However, we here tested a range of response variables expected to be important for soil microbial communities. The M161 genotype was found to produce significantly more root biomass in non-mycorrhizal soil (they also tended to have higher root weight in Experiment 1, even though this was not significant). Divergence from the WT in this way disqualifies M161 from being useful in a WT/ Myc- pair since root abundance is an important contributor to most soil functions, for example soil aggregation (e.g., Six et al. 2002). Additionally, analysis of soil microbial biomass and communities showed that M20 is also not suitable, as these plants gave rise to ~50% greater microbial biomass compared to the WT, presumably due to alteration of root exudate quantity or quality and/ or tissue quality (Lynch and Whipps 1990; Jones et al. 2004).

Other studies have undertaken similar tests in non-mycorrhizal soils. Again, most of the work has been carried out on the *rmc*/ *76R*-WT tomato genotype pair. Most studies have found no difference in growth (Cavagnaro et al. 2004; Poulsen et al. 2005). However, Marschner and Timonen (2005) grew mutant (*rmc*) and WT tomato plants without mycorrhizal inoculum and found differences between the plants. For example, in low light conditions, the *rmc* genotype had approximately half the root mass of the *76R*-WT, with a similar trend also in the high light treatment. This indicates non-target effects that could complicate interpretation of results under certain circumstances. Augé et al. (2004) also explicitly tested Myc- and WT bean plants (*Phaseolus vulgaris*) in non-mycorrhizal

conditions and found no differences in shoot biomass, leaf P content, or soil and leaf water potentials.

Synthesizing results from both our experiments, we can conclude that the MicroTom BC1/WT pair represents a suitable combination for an additional, powerful experimental system for mycorrhizal ecologists. The finding that some mutants we tested were not suitable is at least as important; it illustrates that the clear advantages of employing this and other mutant/ WT pairs can only be fully realized by continuously testing assumptions underlying their use, including tests of new genotypes (as done here), and under different experimental conditions, as has been done with other mutants.

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Figure legends

Fig. 1 Root and shoot dry weights (means and standard errors; n = 10) of different tomato genotypes grown in non-mycorrhizal soil (Experiment 2). For tomato genotype explanations see Table 1.

Fig. 2 Root length and diameter size distribution (means and standard errors; n = 10) of different tomato genotypes grown in non-mycorrhizal soil (Experiment 2). For tomato genotype explanations see Table 1.

Fig. 3 Microbial biomass from the sum of all PLFAs (means and standard errors; n = 5) of different tomato genotypes grown in non-mycorrhizal soil (Experiment 2). For tomato genotype explanations see Table 1.

Fig. 4 Results (PCA) of phospholipid fatty acid (PLFA) analysis of microbial communities resulting from growth in non-mycorrhizal soil (to which soil microbes were added back in a wash; Experiment 2). For tomato genotype (indicated with different symbols and individually labeled) explanations see Table 1.

1 Table 1. Description of tomato (*Solanum lycopersicum*) plants used in the experiments.

2

Genotype	Identity and properties	Reference
WT	Wildtype *Solanum lycopersicum L.*Micro-Tom (miniature tomato cultivar)	Meissner et al. 1997
M161	Pre-mycorrhizal infection mutant (fast neutron mutagenization); single-locus inheritance (recessive). Resistance to infection by spores but not AMF soil hyphae (from neighboring plant) in a controlled environment study (*Glomus intraradices, Gigaspora margarita, Glomus mosseae*)	David-Schwartz et al. 2001, 2003
M20	Pre-mycorrhizal infection mutant (fast neutron mutagenization); Myc- phenotype segregates as a single Mendelian recessive locus (non-allelic to M161); more suppressive Myc- phenotype than M161 in controlled environment study (with *Glomus intraradices*). Seed germination, growth parameters and chlorophyll content was not different from WT.	David-Schwartz et al. 2003
BC1	An F2 segregant of the cross between WT and M161	Y. Kapulnik and P. Bonfante (unpublished)

3

4

5

Table 2 Results (means and standard errors) from growing different tomato genotypes in non-sterile field soil containing AMF (Experiment 1). Different letters within a column indicate differences between means at $P < 0.05$ (Tukey-Kramer HSD or Kruskal-Wallis Z-test, only carried out if overall test was significant). See text for statistical test results.

Tomato genotype	Root weight (g pot^{-1})	Root length (m)	Root length colonized (%)	Root length with arbuscules (%)	Root length colonized (m)	Soil AMF hyphal length (m g^{-1})
WT	0.255 (0.012) a	1.04 (0.06) a	30.3 (7.1) a	17.5 (4.4) a	323.4 (90.0) a	1.16 (0.17) a
M161	0.301 (0.037) a	1.21 (0.23) a	8.6 (4.4) b	7.6 (4.0) b	139.5 (84.9) ab	0.52 (0.06) b
M20	0.249 (0.032) a	1.02 (0.23) a	1.6 (0.5) b	0.1 (0.1) c	14.11 (5.33) b	0.59 (0.10) b
BC1	0.206 (0.025) a	0.89 (0.19) a	1.2 (0.4) b	1.0 (0.5) c	10.54 (3.16) b	0.66 (0.04) b

1 Fig. 1
2

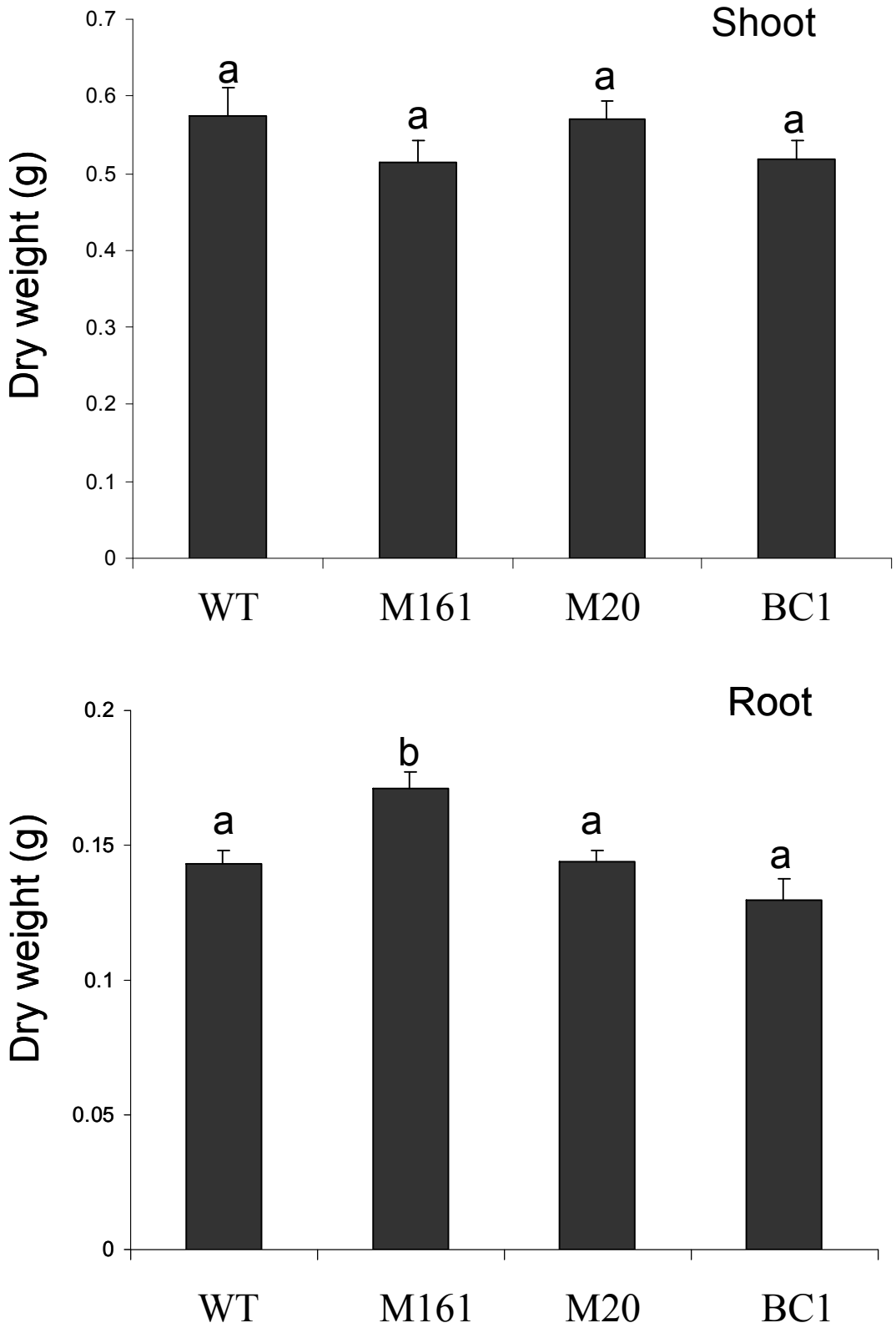

Shoot
Dry weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
a
a
WT
M161
M20
BC1
Root
Dry weight (g)
0.2
0.15
0.1
0.05
0
a
b
a
a
WT
M161
M20
BC1

Fig. 2

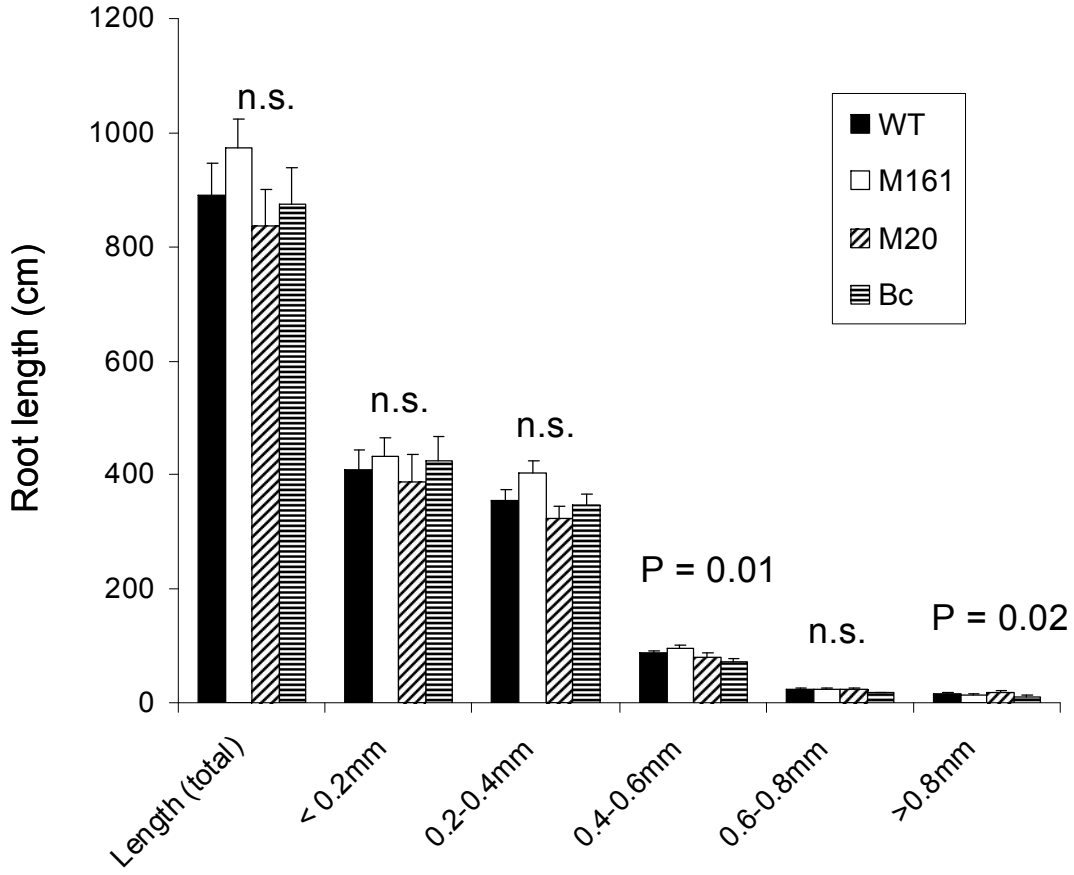

1200
1000
800
600
400
200
0
Root length (cm)
n.s.
n.s.
n.s.
P = 0.01
n.s.
P = 0.02
WT
M161
M20
Bc
Length (total)
< 0.2mm
0.2-0.4mm
0.4-0.6mm
0.6-0.8mm
>0.8mm

Fig. 3

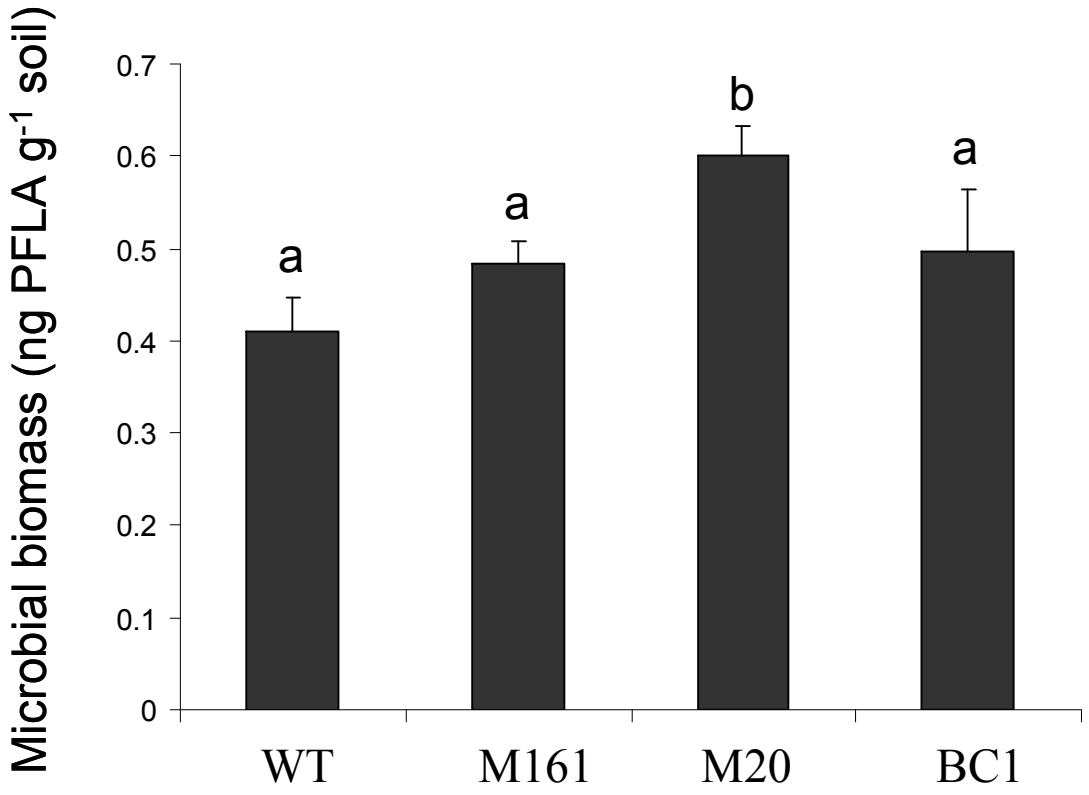
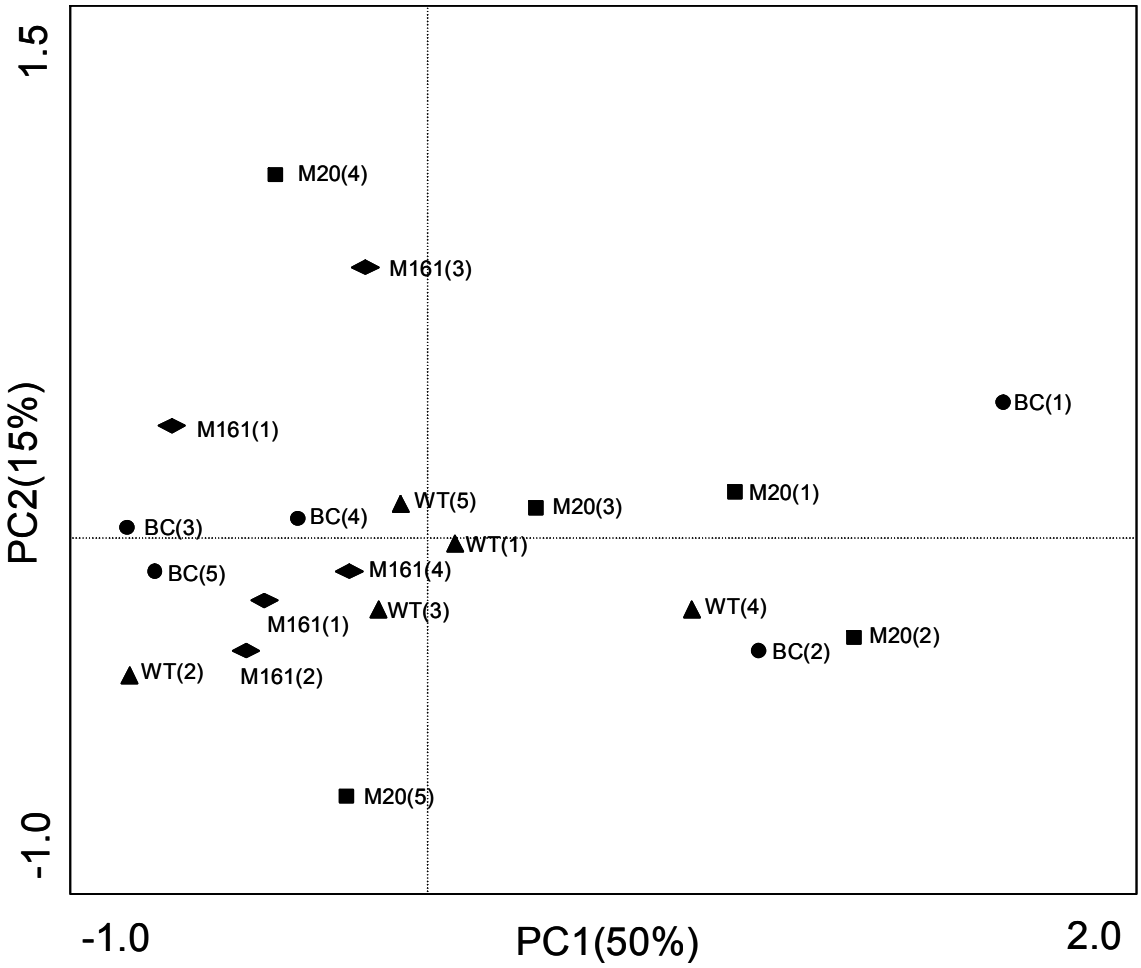

Microbial biomass (ng PFLA g-1 soil)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
b
a
WT
M161
M20
BC1

Fig. 4


1.5
-1.0
PC2(15%)
-1.0
PC1(50%)
2.0
M20(4)
M161(3)
BC(1)
M161(1)
M20(1)
WT(5)
M20(3)
BC(3)
BC(4)
WT(1)
BC(5)
M161(4)
M161(1)
WT(3)
WT(4)
M20(2)
BC(2)
WT(2)
M161(2)
M20(5)