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RESPONSE OF ECTOMYCORRHIZAL FUNGI TO INORGANIC AND ORGANIC FORMS OF NITROGEN AND PHOSPHORUS

Christa M. Luokkala
Michigan Technological University

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By

Christa M. Luokkala

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School of Forest Resources and Environmental Science

Report Advisor: *Dr. Erik A. Lilleskov*

Committee Member: *Dr. Susan A. Bagley*

Committee Member: *Dr. Dana L. Richter*

Committee Member: *Dr. Christopher W. Swanston*

School Dean: *Dr. Terry L. Sharik*

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Abstract

The nutrient uptake response of ectomycorrhizal fungi (ECM) to different nutrient substrates is a driving force in ecosystem nutrient cycling. We hypothesized that taxa from low nitrogen (N) soils would be more likely to use organic N compared to taxa from high N soils, and that taxa from high N would be more likely to use organic phosphorus (P) sources when compared to the ECM dominant in low N soils. This study focuses on the growth response of ECM species collected over a N gradient to different forms of N and P nutrient substrates and whether ECM growth in a particular nutrient source can be related to how the ECM fungi have responded to elevated N in the field. This study found a mixed ECM response to organic and inorganic N and P treatments. High affinity N taxa expected to respond positively to inorganic N produced the phosphatase enzyme to take up organic phosphorus, but not all low affinity N taxa expected to negatively respond to organic P produced the protease enzyme to take up organic N. Interspecific variability was displayed by some high and low affinity N taxa responded and ECM intraspecific variability in response to N and P treatments was also noted. Future analysis of may show more evident ECM response patterns to inorganic and organic forms of N and P.

Preface

This report is planned for publication in an ecological journal. The text was written in first-person plural to give credit to multiple people that have contributed to this research and will be acknowledged as co-authors on the publication.

Introduction

Research is limited on broad spectrum comparisons of ectomycorrhizal fungal (ECM) species utilizing different sources of nitrogen (N) and phosphorus (P). Most studies only compare a few species, and P utilization is poorly understood for many ECM species. Studies have provided data on ECM affinity towards survival under specific concentrations of N and P. Existing literature shows mycorrhizal fungi play a major role in plant N uptake in N-poor environments (Smith & Read 1997). While a higher amount of ECM proficient at producing phosphatase have been identified in areas of higher P immobilization (Conn & Dighton 2000), ECM enzyme production patterns have shown less nitrogenous protein use by dominant ECM from sites of high N concentrations (Taylor et al. 2000, Lilleskov et al. 2002b).

Ectomycorrhizal fungal nutrient uptake

Ectomycorrhizal fungi are known for their influence in producing soluble substrates for cellular assimilation. Ectomycorrhizal fungi can short-circuit the N cycle by directly accessing organic N (amino acids and peptides) via extracellular N-mobilizing enzyme production (Figure 1) (Smith & Read 2008; Chalot & Brun 1998; Näsholm & Persson 2001). Through extracellular enzyme production such as proteases and phosphatases, ECM are capable of improving inorganic N uptake and access to previously unavailable recalcitrant organic N and P for their hosts/symbionts (Read 1991; Chalot & Brun 1998). ECM amino acid cycling is regulated by the presence of a symbiont, N presence, and soil pH levels (Chalot and Brun 1998). The optimality of the ECM extracellular enzyme production and high proteolytic potential is important to consider as soil N availability is altered. Moreover, it is important to consider how ECM under environmental stress can redirect its energy towards optimizing protease and phosphatase production. Pure culture research has shown substantial variation in phosphatase ECM activity (Pacheco et al. 1991).

Nitrogen is one of the main controlling factors in not only tissue growth but also organismal survival. In forest soils, over 95% of soil N is in organic form and less than

5% of total N in soil is in inorganic ammonium (NH_4^+) and nitrate (NO_3^-) forms. Soil N locked up in organic forms requires ECM proteolytic enzyme production to access this N pool (Chalot & Brun 1998).

Ectomycorrhizal fungal species differ in their response to elevated N. Increased N over time causes ECM sporocarp production to change (Lilleskov et al. 2001) and influences community structure (Lilleskov et al. 2001 and 2002a). Considerable attention has been given to understanding how different ECM species respond to varying concentrations of N and different forms of N (Lilleskov et al. 2011 and references therein).

Phosphorus is also a limiting nutrient due to its role in nutrient transport and energy transfer. Ectomycorrhizal hyphal patches are sinks for P (phosphate fed into the growing hyphae) (Read 1991), and when P substrate levels decrease, the enzyme group acid phosphatases are activated in ECM to facilitate P uptake (Pacheco, et al. 1991). Research is lacking concerning the role different forms and availability of P influence ECM species development and dominance (Marschner and Dell 1994; Treseder 2004).

Another consideration in ECM survival and/or dominance is the form of nutrients available for the ECM to take up. There appears to be no studies simultaneously comparing ECM species preference for inorganic and organic forms of both N and P. N and P must be present in suitable concentrations to provide the building blocks for cellular growth and repair. Inorganic N occurs as ammonium (NH^{4+}) that dominates in acid soils and nitrate (NO_3^-) (Brady and Weil 2008). Organic N, which is the dominant form of N in soil, includes proteins (amino acids and peptides) that most ECM are capable of taking up by extracellular enzyme production (Smith and Read 2008). Inorganic P is predominantly present in the form of phosphate (PO_4^-) and has been shown in high amounts to inhibit phosphatase production in ECM (Sinsabaugh and Linkins 1992). Organic P is ubiquitous across all environments primarily in a variety of diester and monoester bonded phosphate groups. Monoester bonded phosphates, including inositol phosphates, require ECM to produce phosphomonoesterase enzymes to facilitate the uptake of P (Turner et al. 2002, Read and Perez-Moreno 2003). N and P forms can be

present in varying amounts that may lead to interspecific variation and specialization in how fungal species respond to different forms of N and P.

Ectomycorrhizal fungal culturing

Nutrient uptake by ECM has received critical attention on a species by species basis (Jentschke et al. 2001, Read and Perez-Moreno 2003, Treseder 2004). However, laboratory experiments quantifying nutrient uptake affinities by ECM collected from nutrient gradients are very rare in the literature (Wallenda and Read 1999). One reason for the lack of reports comparing interspecific species responses to different forms of nutrients is due to the vast preparatory work involved in such experiments. ECM fungal collecting involves a basic knowledge on ECM sporocarp and root tip morphology, a system to keep track of the reference fruiting body along with storage space to incubate live cultures, and the ability to extract tissue from the sporocarp or root tip to plate on agar plates under sterile conditions. ECM tissue growth involves an extensive amount of labor and the continuous concern over sterile workplace conditions. ECM identification is a step-by-step process requiring skilled labor for confirming culture viability and advanced molecular techniques to confirm the identification and lack of contamination of a fungal isolate (Heinonen-Tanski and Holopainen 1991; Rygiewicz and Armstrong 1991; Smith and Onions 1994). After all of this, an experiment can finally be conducted on the viable, uncontaminated, and identified fungi.

The most ideal medium form for culturing ECM after identification is in liquid culture tubes due to the reduced surface area exposed to the environment, the conservation of laboratory space, and the eliminated occurrence for dehydration. Liquid culture assists in reducing the growth substrate heterogeneity typical of soil. Repeated measurements on single root tips can be assayed over time with reproducibility and no loss of enzyme activity, because there are membrane-bound enzymes on the ECM. Thus, enzymes are not lost over repeated measures when transferred from one liquid substrate to the next (Pritsch, et al. 2004). Several ECM including *Thelephora*, *Clitocybe*, *Tricholoma*, and *Hebeloma* grow well after storage in sterile water, and *Leccinum* and *Suillus* had 100% viability after storage (Richter and Brun 1989). Richter (2008) has also

shown ECM are viable after being stored in sterile water for twenty years. Furthermore, culturing ECM in test tubes containing liquid media eliminates the necessity for measuring evaporation from dishes (Rangel-Castro et al. 2002).

Study description

We chose ECM fungal taxa from across a N gradient to investigate their growth response to inorganic N, organic N, inorganic P, and organic P sources. This report analyzes eight ECM taxa from this study. Intraspecific ECM physiological variation can be underestimated if only one strain of each species is used experimentally (Cairney 1999). There is some understanding on how these fungal isolates respond to different forms of inorganic and organic N and P (Table 1). This study represents a broad range of ECM fungi in temperate forest soils. We chose several species to represent different N and P uptake capabilities. *Paxillus involutus* is an ideal ECM for in vitro experimentation due to its fast growth. Less is known about ECM more difficult to grow in pure culture such as *Cortinarius, Lactarius, Russula, Tomentella,* and *Tricholoma* (Nygren et al. 2007).

The overall study examines the growth response of 24 ECM species collected over a N gradient to different forms of N and P and whether ECM growth in a particular nutrient source can be related to how the ECM fungi have responded to elevated N in the field (Table 3). The primary objective of this study focuses on the growth response of ECM species to different nutrient substrates. Deciphering ECM response to nutrient sources will improve understanding of the internal N cycling which is the critical driver of ECM community change. The secondary objective of this study delves into inter- and intraspecific variation in growth on different N and P forms depending on the N availability at the site of origin. *Hypotheses: The ectomycorrhizal fungi from low N soils will utilize organic N more efficiently than ectomycorrhizal fungi from high N soils. In contrast, the ectomycorrhizal fungi from high N soils will utilize organic P more efficiently than ectomycorrhizal fungi from low N soils.*

Materials and Methods

Study area

The study sites were located on the Kenai Peninsula, in Alaska, USA (Figure 2 and for details of study area see Lilleskov et.al. 2001, 2002a, and 2002b). An atmospheric ammonium deposition gradient exists near a fertilizer manufacturing facility with increased levels of N deposition, resulting in higher soil inorganic N availability (Lilleskov et.al. 2002a). Sites were established in 1994 and are dominated by *Picea glauca* (white spruce) and *Betula kenaica* (Kenai paper birch) on well drained, fine sandy loam glacial outwash (Lilleskov et al. 2001).

Field sampling and direct sequence analysis

Sporocarps and root tips were collected by Erik Lilleskov in August 2006 and 2007 at five sites from across this gradient. Sporocarps and root tips were located at each site in close proximity to mature white spruce trees. Sporocarps previously identified in Lilleskov et al. (2001) were collected preferentially. Sections of forest floor were taken back to the lab for cleaning and dissection. Root tips were sorted morphologically under a dissecting microscope. Clean internal sporocarp tissue or hydrogen peroxide sterilized EMF root tip samples were transferred using sterile technique to Petri plates with Modified Melin Norkrans (MMN) agar (Marx 1969) containing benomyl fungicide and antibiotics. Voucher specimens of sporocarps were established at the time of collection and are stored at the USDA Forest Service Northern Research Station, Houghton, MI, USA. After growth was established on the MMN agar or liquid, 125 sporocarp and root tip cultures were selected in accordance to their successful growth and site location (low, intermediate, and high) over the N gradient. Identification of the 125 isolates was confirmed using molecular genetic sequencing following the methods of Andrew (2009) (Luokkala, unpublished data).

Experimental isolate preparation

The cultures were stored at 15°C on MMN agar or MMN liquid while fast growing cultures such as *Paxillus involutus* were stored as 5-mm-diameter plugs in sterile distilled deionized water (ddH_2O) and slow growing cultures such as *Piloderma* spp. were placed in citrate buffer solution for approximately one year at the US Forest Service Northern Research Station Forestry Sciences Laboratory, Houghton, MI, USA (Table 3). The cultures selected for this experiment (Table 3) were removed from storage and checked for viability by transferring them into fresh MMN media for approximately six months (some faster growing isolates were transferred several times). Once viability was confirmed, residual agar was carefully removed from the experimental isolates and they were placed in jars of induction medium where their tissue was broken up with a sterile immersion blender allowing for a homogenous hyphal distribution in solution (Appendix 1). The basal induction medium was 10% strength MMN (Kottke et al. 1987) excluding malt extract and ammonium phosphate. Ammonium chloride (NH_4Cl) replaced the ammonium phosphate nitrogen source (N:P = 10), citrate buffer was added to maintain the medium at pH 4.8, and glucose was added as the sole carbon source (Appendix 1, 4, and 5). The isolates remained in the induction medium for approximately two months in static culture at 15°C.

Pure culture N and P experiment

The experimental cultures were grown in test tubes without N (minus-N control), with ammonium chloride (NH_4Cl) for the inorganic N treatment, with essentially globulin and protease free albumin from bovine serum (BSA, #A3059 Sigma-Aldrich Co., St. Louis, MO, USA) for the organic N treatment, without P for the minus-P control, with potassium phosphate (KH_2PO_4) for the inorganic P treatment, and with phytic acid dipotassium salt ($C_6H_{16}K_2O_{24}P_6$, #P5681 Sigma-Aldrich Co., St. Louis, MO, USA) for the organic P treatment. For organic N and P assays, the basal medium for the experiment was MMN excluding malt extract with glucose added at 5 g/L serving as the carbon source (Appendices 2-4). The citrate buffer was again used across all treatments to maintain pH at 4.8.

Bovine serum albumin, representing a large group of water soluble proteins found in plants and animals, is an omnipresent proteinaceous nitrogen source used in mycorrhizal fungal proteinase incubation experiments (Abuzinadah and Read 1986, Leake and Read 1991). The phytic acid dipostassium salt was qualitatively assayed to confirm its purity in Dr. Pushpalatha Murthy's chemistry lab, Michigan Technological University, Houghton, MI, USA. The BSA and phytic acid were filter sterilized before each one was added to the autoclaved basal medium.

Each inoculum was equally pipetted at 1 mL from the induction jars into their respective test tubes (n=3). There were three harvest dates for each treatment with three replications on each harvest date. All initial harvest dates were conducted on day 24 or 25 after inoculation. Cultures were harvested, vacuum filtered, freeze dried, and fungal biomass was measured on a microbalance (Mettler-Toledo XP56). The second and third harvest dates were executed according to visible fungal growth rates in an attempt to capture a representative growth rate according to the fungal response to each of the treatments. See Appendix 5 for detailed experimental protocol.

Statistical design and analysis

The study tested fungal growth in response to inorganic and organic N and P nutrient treatments relative to minus-N and minus-P controls. The overall study independent variables were the fungal species, strains of fungal isolates within species, the site of isolate origin (low, intermediate, and high N), the N and P nutrient treatments (inorganic, organic, and control), and time (1, 2, and 3 harvests). Some species were not found at all sites. A harvest of each inoculum at each harvest prevented a repeated measures approach. The dependent variable was fungal biomass.

This report only tested the independent variables N and P nutrient treatments (inorganic, organic, and control) and the variable time (1, 2, and 3 harvests). The fungal growth in the N treatments relative to minus-N controls and in the P treatments relative to the minus-P controls were tested using one-way ANOVAs. A Dunnett's post hoc comparison test was utilized to compare mean treatment affects relative to control at *p*

values at 0.05, 0.01, and 0.001 (Zar 1999). All statistical tests were done in Minitab 15 (Minitab Inc. 2012).

Results

Nitrogen utilization

All of the ECM species analyzed in this report had significant growth on inorganic N ($p < 0.05$), while selected isolates of four species (*P. involutus*, *P. byssinum*, *P. fallax*, and *L. bicolor*) from across the N gradient significantly responded to organic N ($p < 0.05$) (Tables 4 and 8). *Paxillus involutus* isolate 07-29B, *P. byssinum* isolate 749, and *A. byssoides* isolate 873 did not display a significant growth response in the inorganic and organic N treatments ($p > 0.05$) (Figure 7 and Table 4). Consistent with our hypothesis, two low affinity N taxa (*P. byssinum* and *P. fallax*) displayed significant growth on protein (Figures 14 and 16 and Table 4). However, two high affinity N taxa (*L. bicolor* and *P. involutus*) also grew on the protein (Figures 4 and 5 and Table 4). High affinity taxon *P. involutus* isolates and low affinity N taxon *P. fallax* isolates showed significant intraspecific variation in response to the organic N treatment ($p < 0.05$) (Figures 4, 6, 8, 9, and 13-15 and Table 4).

Growth patterns varied over the harvest dates for some isolates. *Laccaria bicolor* significantly responded to both inorganic and organic N treatments ($p < 0.0001$ for both treatments at the second and third harvests). While its growth on organic N lagged behind the growth on inorganic N by 18.26 ± 1.60 mg (mean ± 1 SE) at the second harvest, growth drastically slowed down on inorganic N at the third harvest by 0.80 ± 1.82 mg displaying growth on organic N was similar to the biomass on inorganic N (Figure 5 and Table 4). *Piloderma byssinum* isolate 287 displayed a similar growth trajectory in both inorganic and organic N treatments ($p < 0.05$) (Figure 16 and Table 4). In contrast, *P. involutus* isolate 06-38 displayed significant growth on organic N that lagged behind the growth on inorganic N in relation to the control by 19.14 ± 1.08 mg at the third harvest ($p < 0.05$) (Figure 4 and Table 4). Although the two treatments were insignificant relative to the control, *P. involutus* isolate 07-29B displayed a growth trend

on organic N that lagged behind the growth on inorganic N by 11.91 ± 8.29 mg at the second harvest and only lagged behind by 6.21 ± 11.94 mg at the third harvest date (Figure 7 and Table 4).

Phosphorus utilization

At least one isolate of each of the ECM species except *P. byssinum* grew significantly on inorganic P ($p < 0.05$) (Tables 5 and 8). Fewer isolates responded positively to the organic P treatment (Tables 5 and 8). Low affinity N taxon *P. fallax* isolate 18 and the high affinity N taxon *P. involutus* isolate 862 only significantly grew on inorganic P in respect to the control (Figures 10 and 15 and Table 5).

Isolates from the P treatments in which three harvest dates were analyzed in this report displayed intraspecific and interspecific variation in growth patterns. One distinctive pattern was in high N affinity isolates *P. involutus* isolates 06-38 and 07-20D and Atheliaceae 1 642. They displayed a growth pattern in which the growth rate on inorganic P was faster, the organic P uptake was more gradual, and the biomass accumulation was significantly higher in the organic P treatment at the third harvest date ($p < 0.05$) (Figures 3, 4, and 6 and Table 5). This growth trend was also noted in two low N affinity isolates *P. fallax* 16 and *A. byssoides* 873 both from low N sites (Figures 14 and 20). In contrast, high N affinity isolates *P. involutus* 07-29B displayed an opposite growth pattern in which the growth rate on organic P was higher, the inorganic P uptake was more gradual, and the biomass accumulation was higher in the inorganic P treatment at the third harvest date (Figures 12 and Table 5).

Discussion

Nitrogen utilization

The first hypothesis expecting taxa from low N soils to utilize organic N more efficiently than taxa from high N soils was not supported by all of the isolates from across the gradient. Additionally, one isolate from an intermediate N site (*L. bicolor*) and another isolate from a high N site (*P. involutus*) grew on protein, which reduces the

supposition that taxa from low N sites would show a greater capability in producing proteases for the uptake of organic nitrogen sources. However, ECM have displayed reduced protein N uptake under higher N deposition (Lilleskov et al. 2002b, Taylor et al. 2000). Our results showed two (*P. byssinum* and *P. fallax*) out of the five species from low N sites responded to the organic N treatment (*A. byssoides*, *H. speciosus*, and *P. involutus* did not respond), while only 22% of the total isolates from the low N sites responded to the organic N treatment indicating intraspecific variation.

Interestingly, low and high affinity N taxa both responded to the protein treatments in a similar manner showing there is greater interspecific variability than the hypothesis took into account. It appeared as though the isolates *L. bicolor* 06-64.1, *P. involutus* 07-20D, and *P. involutus* 07-29B displayed a lag growth and eventually acclimatized to the treatments to actively produce protease enzymes to take up organic nitrogen, while other species such as *A. byssoides*, *P. fallax*, *P. byssinum*, and *H. speciosus* were able to grow on both inorganic and organic N nutrients without a delayed response time. Interestingly, accumulated research shows the genus *Laccaria* has proven to be defined as a nonuser of protein N (Lilleskov 2011), but this analysis has found it has significantly responded to organic N. It is important to point out that *L. bicolor's* growth on protein N substrate showed up only after 50 days of incubation (Figure 5 and Table 4).

The data has also shown intraspecific variability in response to nitrogen amongst the *P. involutus*, *A. byssoides*, and *P. fallax* isolates which the hypothesis does not take into account. *Paxillus* have shown to be variable protein users while *Piloderma* have been cited as protein users (Lilleskov 2011). However, the results in this study place *P. fallax* as a variable user. This analysis has also found that previously undefined *Amphinema* and *Hygrophorus* as potential protein users. One of the variables that should be considered is the amount of time cultures are allowed to grow.

Phosphorus utilization

The data did not fully support the second hypothesis regarding taxa from high N sites should be expected to use organic P sources compared to the ECM dominant in low N soils, because there was not a difference in the overall species response to inorganic

and organic phosphorus nutrient treatments. However, this data set ascertains many and/or most ECM are capable of inositol hexaphosphate breakdown and subsequent uptake.

Even though all of the isolates analyzed in this report positively responded to the organic P treatment, it is important to point out that the isolate site location might play a role in the distinctive and contrasting growth rate patterns. The isolates that displayed an initial higher growth rate on organic P were from the high N site while the isolates that displayed a lagged growth response to organic P while quickly growing on inorganic P were from the low N sites. This could indicate intraspecific variability amongst the isolates from high N sites requiring less time to be induced into producing phosphatase enzymes for organic P uptake compared to the isolates from low N sites.

Conclusion

In conclusion, the resulting data for both N and P treatments displays a mixture of support, refutation, and mixed results in respect to the hypotheses. High affinity N taxa expected to respond positively to inorganic N produced the phosphatase enzyme to take up organic phosphorus, but not all low affinity N taxa expected to negatively respond to organic P produced the protease enzyme to take up organic N. Interestingly, some high and low affinity N taxa responded in nearly an identical manner to some of the N and P treatments signifying interspecific variability. Lastly, this analysis has also shown ECM intraspecific variability in response to N and P treatments. Future analysis of the remaining data may show more evident ECM response patterns to the inorganic and organic forms of N and P.

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Tables

Table 1
The documented ectomycorrhizal fungal response to elevated nitrogen of the taxa analyzed in this report (Lilleskov et al. 2011).

Low	High
Amphinema	*Atheliaceae 1*
Hygrophorus	*Laccaria*
Thelephora	*Paxillus*
	Piloderma

Table 2
Ectomycorrhizal fungal growth response to protein (Lilleskov et al. 2011).

Nonprotein Users	**Variable Protein Users**	**Protein Users**	No Data
Laccaria	*Cenococcum* *Lactarius* *Paxillus* *Thelephora* *Tylospora*	*Cortinarius* spp. *Piloderma* *Russula* *Tricholoma*	*Amphinema* *Hygrophorus* *Pseudotomentella* *Tomentella*

Table 3

Fungal species used in the study with details of their isolation from N deposition site origin. The taxa in bold were analyzed in this report for growth response on inorganic and organic nitrogen and phosphorus nutrient treatments.

Experimental Species	Low N	Intermediate N	High N
Atheliaceae 1 Jülich	**x**	**x**	**x**
***Amphinema byssoides* (Pers.:Fr.) Erikss.**	**x**	**x**	**x**
Cortinarius sp. (Pers.) Gray	x		x
Cenococcum geophilum Fr.	x		
Hygrophorus pustulatus (Pers. Ex Fr.) Fr.	x		
***Hygrophorus speciosus* Pk.**	**x**		
***Laccaria bicolor* (Maire) Orton**		**x**	**x**
Lactarius olivaceoumbrinus Smith			x
Lactarius tabidus Fr.			
Lactarius theiogalus (Bull Fr.) S.F. Gray	x	x	x
***Paxillus involutus* (Batsch : Fr.) Fr.**	**x**	**x**	**x**
***Piloderma byssinum* (Karst.) Jülich**	**x**		
***Piloderma fallax* (Libert) Stalpers**	**x**		
Pseudotomentella sp. Svrcek	x		
Russula bicolor Burlingham	x		
Russula spp. Pers.	x	x	x
Russula claroflava Grove			x
***Thelephora terrestris* Fr.**		**x**	**x**
Tomentella spp. Pers.ex Pat.	x		
Tomentella stuposa (Link) Stalpers	x		
Tomentella sublilacina (Ellis & Holw.) Wakef.		x	x
Tricholoma platyphyllum (Murrill) Sacc. & Trotter	x		
Tricholoma inamoenum (Fr.:Fr.) Gill.	x		
Tricholoma saponaceum (Fr.) Kummer	x		
Tylospora fibrillosa (Burt) Donk		x	

Table 4

Fungal species from either sporocarp or white spruce (*Picea glauca*) root tip from a N deposition site in-vitro growth response to inorganic and organic N treatments. Biomass (mg) difference between treatments and a control (mean ± SE). n=3

			Harvest 1		Harvest 2		Harvest 3	
Species	Isolate	Site	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
Amphinema byssoides	934	L	**0.80 ± 0.65***	-0.53 ± 0.41	**3.44 ± 0.82****	1.63 ± 0.73	**8.22 ± 0.70****	5.42 ± 2.39
Hygrophorus speciosus	740	L	0.38 ± 0.34	**1.19 ± 0.49**	**15.82 ± 3.51***	7.62 ± 3.75	n/a	n/a
Paxillus involutus	862	L	**2.58 ± 4.60**	0.05 ± 1.13	**8.87 ± 6.50**	2.43 ± 0.95	1.61 ± 4.38	**3.30 ± 2.08**
Piloderma byssinum	287	L	0.54 ± 0.71	**1.02 ± 0.31**	1.54 ± 0.42*	**1.74 ± 0.59***	n/a	n/a
Piloderma byssinum	749	L	**1.03 ± 0.39**	0.24 ± 0.41	**3.36 ± 2.10**	2.33 ± 1.25	n/a	n/a
Piloderma fallax	14	L	**1.10 ± 0.38**	0.93 ± 0.55	**10.25 ± 3.25****	1.36 ± 1.65	n/a	n/a
Piloderma fallax	16	L	**2.47 ± 0.28*****	1.49 ± 0.28**	3.01 ± 2.01	**3.87 ± 2.15**	n/a	n/a
Piloderma fallax	18	L	**0.60 ± 0.62**	0.06 ± 0.79	**6.89 ± 2.42***	2.23 ± 0.44	n/a	n/a
Piloderma fallax	157	L	**1.21 ± 0.83**	0.49 ± 0.51	**11.54 ± 1.33*****	0.73 ± 1.01	**12.77 ± 5.44***	0.84 ± 0.52
Amphinema byssoides	873	I	**0.35 ± 1.18**	-0.36 ± 0.30	**3.80 ± 3.73**	0.15 ± 0.60	n/a	n/a
Laccaria bicolor	06-64.1	I	**6.79 ± 0.99*****	3.51 ± 0.70*	**22.71 ± 0.60*****	4.45 ± 1.48*	**24.84 ± 0.37*****	24.04 ± 1.78***
Paxillus involutus	07-20D	I	0.44 ± 0.71	**0.45 ± 0.95**	**4.71 ± 2.29****	1.78 ± 2.14	**7.58 ± 0.95*****	4.16 ± 0.69***
Thelephora terrestris	489a	I	**0.83 ± 0.41**	0.12 ± 0.27	**18.32 ± 5.44****	0.75 ± 0.64	n/a	n/a
Atheliaceae 1	642	H	**3.60 ± 0.37***	0.26 ± 0.44	**8.74 ± 2.10****	-0.80 ± 0.70	**17.48 ± 2.93*****	0.84 ± 1.36
Paxillus involutus	06-38	H	**5.30 ± 2.98**	-0.47 ± 1.14	**8.50 ± 5.94**	1.23 ± 0.22	**21.70 ± 1.01*****	2.56 ± 0.37*
Paxillus involutus	06-40	H	0.22 ± 4.01	**0.97 ± 0.11**	0.02 ± 0.55*	**0.57 ± 0.98**	n/a	n/a
Paxillus involutus	07-29B	H	**3.15 ± 3.11**	0.93 ± 0.83	**12.94 ± 8.23**	1.03 ± 0.98	**15.01 ± 10.12**	8.80 ± 6.34
Thelephora terrestris	644	H	**3.89 ± 1.56**	0.26 ± 0.28	**18.98 ± 8.97***	0.34 ± 2.39	**13.24 ± 3.31****	0.87 ± 0.83

Note: Asterisks indicate significant growth relative to control (*P < 0.05, **P < 0.01, and ***P < 0.001 for all comparisons for that species on that date). Bolded treatment at each harvest indicates the treatement with the greater change in biomass.

Table 5

Fungal species from either sporocarp or white spruce (*Picea glauca*) root tip from a N deposition site in-vitro growth response to inorganic and organic P treatments. Biomass (mg) difference between treatments and a control (mean ± SE). n=3

			Harvest 1		Harvest 2		Harvest 3	
Species	Isolate	Site	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
Amphinema byssoides	934	L	0.52 ± 0.47	**0.85 ± 0.39**	**2.42 ± 0.83**	1.90 ± 1.09	**9.60 ± 0.75*****	7.84 ±0.93***
Hygrophorus speciosus	740	L	**2.05 ± 0.91**	0.91 ± 0.82	10.75 ± 0.91***	**13.27 ± 1.14*****	n/a	n/a
Paxillus involutus	862	L	**3.83 ± 5.28**	3.80 ± 5.61	7.30 ± 5.62	**7.58 ± 5.82**	**15.7 ± 4.44***	9.70 ± 5.52
Piloderma byssinum	287	L	**0.19 ± 0.85**	-0.47 ± 0.42	**3.18 ± 1.78**	2.98 ± 1.41	n/a	n/a
Piloderma byssinum	749	L	-0.48 ± 0.16	**0.50 ± 0.76**	1.19 ± 0.48	**5.05 ± 3.29**	n/a	n/a
Piloderma fallax	14	L	0.81 ± 1.16	**1.03 ± 1.06**	11.57 ± 2.99**	**17.21 ± 1.58****	n/a	n/a
Piloderma fallax	16	L	**1.52 ± 0.75**	0.14 ± 0.12	**7.48 ± 5.60**	2.12 ± 2.15	n/a	n/a
Piloderma fallax	18	L	0.30 ± 0.16	**0.56 ± 0.68**	**9.54 ± 2.60****	1.40 ± 0.81	n/a	n/a
Piloderma fallax	157	L	1.14 ± 1.21	**2.13 ± 1.45**	6.35 ± 1.02**	**9.27 ± 1.79*****	15.37 ± 1.86***	**18.78 ± 1.42*****
Amphinema byssoides	873	I	**0.77 ± 0.60**	-0.21 ± 0.55	1.76 ± 2.19	**3.05 ± 1.69**	n/a	n/a
Laccaria bicolor	06-64.1	I	**7.41 ± 2.33**	3.17 ± 3.01	**23.22 ± 2.13*****	20.57 ± 1.81***	21.72 ± 0.73***	**24.51 ± 1.42*****
Paxillus involutus	07-20D	I	1.33 ± 0.45*	**2.83 ± 0.55*****	1.57 ± 1.35	**2.05 ± 0.11**	4.23 ± 3.40*	**5.24 ± 3.00****
Thelephora terrestris	489a	I	**2.96 ± 0.33****	1.58 ± 0.65	13.25 ± 3.89*	**14.02 ± 4.70***	n/a	n/a
Atheliaceae 1	642	H	0.19 ± 0.45	**0.92 ± 0.75**	**14.34 ± 1.77*****	7.66 ± 1.20**	**13.21 ± 2.46*****	10.68 ± 1.66**
Paxillus involutus	06-38	H	7.83 ± 2.57	**10.76 ± 2.58***	**14.57 ± 4.40**	8.45 ± 7.11	**19.14 ± 2.02****	15.09 ± 5.90**
Paxillus involutus	06-40	H	0.08 ± 0.29	**6.52 ± 4.03**	**12.96 ± 6.43**	11.59 ± 6.48	n/a	n/a
Paxillus involutus	07-29B	H	0.01 ± 0.93	**2.25 ± 2.46**	2.56 ± 1.79	**11.81 ± 6.08**	**21.17 ± 0.80**	10.90 ± 9.90
Thelephora terrestris	644	H	5.40 ± 2.05	**5.42 ± 2.71**	18.66 ± 8.13	**20.52 ± 2.29**	**18.94 ± 1.10*****	18.23 ± 0.31***

Note: Asterisks indicate significant growth relative to control (*P < 0.05, **P < 0.01, and ***P < 0.001 for all comparisons for that species on that date). Bolded treatment at each harvest indicates the greater change in biomass between the inorganic and organic treatments.

Table 6

Dunnett's test *P* values for fungal species from either sporocarp or white spruce (*Picea glauca*) root tip from a N deposition site in-vitro growth response to inorganic and organic N between treatments and a control. n=3

			Harvest 1		Harvest 2		Harvest 3	
Species	Isolate	Site	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
Amphinema byssoides	873	I	0.91	0.90	0.40	1.00	n/a	n/a
Amphinema byssoides	934	L	0.03	0.55	0.01	0.12	0.01	0.06
Hygrophorus speciosus	740	L	0.61	0.06	0.02	0.20	n/a	n/a
Laccaria bicolor	06-64.1	I	0.0008	0.02	<0.0001	0.02	<0.0001	<0.0001
Paxillus involutus	06-38	H	0.12	0.97	0.22	0.95	<0.0001	0.04
Paxillus involutus	06-40	H	0.22	0.98	0.02	0.57	n/a	n/a
Paxillus involutus	07-20D	I	0.79	0.78	0.01	0.31	<0.0001	0.001
Paxillus involutus	07-29B	H	0.42	0.91	0.17	0.98	0.18	0.47
Paxillus involutus	862	L	0.62	0.10	0.18	0.83	0.71	0.31
Piloderma byssinum	287	L	0.61	0.24	0.03	0.02	n/a	n/a
Piloderma byssinum	749	L	0.12	0.82	0.10	0.25	n/a	n/a
Piloderma fallax	14	L	0.14	0.22	0.01	0.77	n/a	n/a
Piloderma fallax	16	L	0.0004	0.005	0.40	0.25	n/a	n/a
Piloderma fallax	18	L	0.54	0.99	0.02	0.46	n/a	n/a
Piloderma fallax	157	L	0.17	0.67	<0.0001	0.51	0.05	0.97
Thelephora terrestris	489a	I	0.09	0.91	0.01	0.98	n/a	n/a
Thelephora terrestris	644	H	0.06	0.97	0.03	0.10	0.003	0.92
Atheliaceae 1	642	H	0.04	0.96	0.004	0.86	0.0003	0.89

Table 7

Dunnett's test *P* values for fungal species from either sporocarp or white spruce (*Picea glauca*) root tip from a N deposition site in-vitro growth response to inorganic and organic P between treatments and a control. n=3

			Harvest 1		Harvest 2		Harvest 3	
Species	Isolate	Site	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
Amphinema byssoides	873	I	0.33	0.90	0.45	0.15	n/a	n/a
Amphinema byssoides	934	L	0.37	0.12	0.12	0.21	<0.0001	0.0001
Hygrophorus speciosus	740	L	0.13	0.57	0.0002	0.0001	n/a	n/a
Laccaria bicolor	06-64.1	I	0.09	0.51	0.0001	0.0002	<0.0001	<0.0001
Paxillus involutus	06-38	H	0.06	0.02	0.09	0.33	0.005	0.01
Paxillus involutus	06-40	H	1.00	0.16	0.17	0.22	n/a	n/a
Paxillus involutus	07-20D	I	0.04	0.001	0.92	0.36	0.03	0.01
Paxillus involutus	07-29B	H	1.00	0.36	0.82	0.09	0.07	0.36
Paxillus involutus	862	L	0.49	0.48	0.41	0.38	0.02	0.12
Piloderma byssinum	287	L	0.92	0.63	0.20	0.23	n/a	n/a
Piloderma byssinum	749	L	0.65	0.63	0.86	0.16	n/a	n/a
Piloderma fallax	14	L	0.44	0.30	0.01	0.002	n/a	n/a
Piloderma fallax	16	L	0.07	0.96	0.11	0.76	n/a	n/a
Piloderma fallax	18	L	0.81	0.52	0.004	0.69	n/a	n/a
Piloderma fallax	157	L	0.68	0.33	0.01	0.001	0.0001	<0.0001
Thelephora terrestris	489a	I	0.01	0.06	0.04	0.04	n/a	n/a
Thelephora terrestris	644	H	0.09	0.09	0.11	0.79	<0.0001	<0.0001
Atheliaceae 1	642	H	0.93	0.29	0.0002	0.006	0.0009	0.003

Table 8

A summary of the ectomycorrhizal significant growth response to inorganic and organic nitrogen and phosphorus in comparison to the controls ($p < 0.05$).

Species	Isolate	Site	Inorganic N	Organic N	Inorganic P	Organic P
Amphinema byssoides	934	L	X_a		X_3	X_3
Hygrophorus speciosus	740	L	X_2		X_2	X_2
Paxillus involutus	862	L			X_3	
Piloderma byssinum	287	L	X_2	X_2		
Piloderma byssinum	749	L				
Piloderma fallax	14	L	X_2		X_2	X_2
Piloderma fallax	16	L	X_1	X_1		
Piloderma fallax	18	L	X_2		X_2	
Piloderma fallax	157	L	$X_{2,3}$		$X_{2,3}$	$X_{2,3}$
Amphinema byssoides	873	I				
Laccaria bicolor	06-64.1	I	X_a	X_a	$X_{2,3}$	$X_{2,3}$
Paxillus involutus	07-20D	I	X_2		$X_{1,3}$	$X_{1,3}$
Thelephora terrestris	489a	I	X_2		$X_{1,2}$	X_2
Atheliaceae 1	642	H	X_a		$X_{2,3}$	$X_{2,3}$
Paxillus involutus	06-38	H	X_3	X_3	X_3	$X_{1,3}$
Paxillus involutus	06-40	H	X_2			
Paxillus involutus	07-29B	H				
Thelephora terrestris	644	H	$X_{2,3}$		X_3	X_3

a indicates significance at harvest 1, 2, and 3; 1 indicates significance at harvest 1; 2 indicates significance at harvest 2; 3 indicates significance at harvest 3

Figures

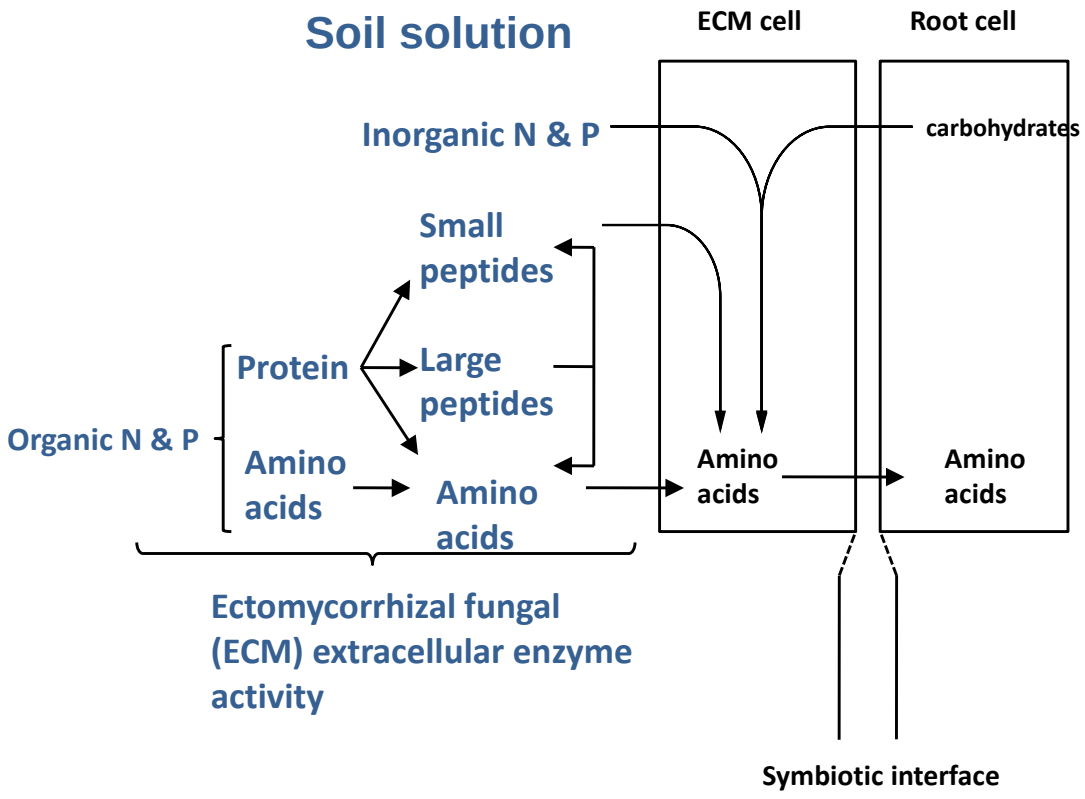


Figure 1 A model of the soil matrix, ectomycorrhizal fungal, and root interfaces (modified from Chalot and Brun 1998). Inorganic nitrogen and phosphorus are available for ectomycorrhizal fungi and plants to directly take up. Organic nitrogen and phosphorus bound in protein and amino acids are hydrolyzed by extracellular enzymes produced by ectomycorrhizal fungi which then take up the accessible nutrients. The nutrients taken up by the fungi are exchanged for carbohydrates from the plant root.

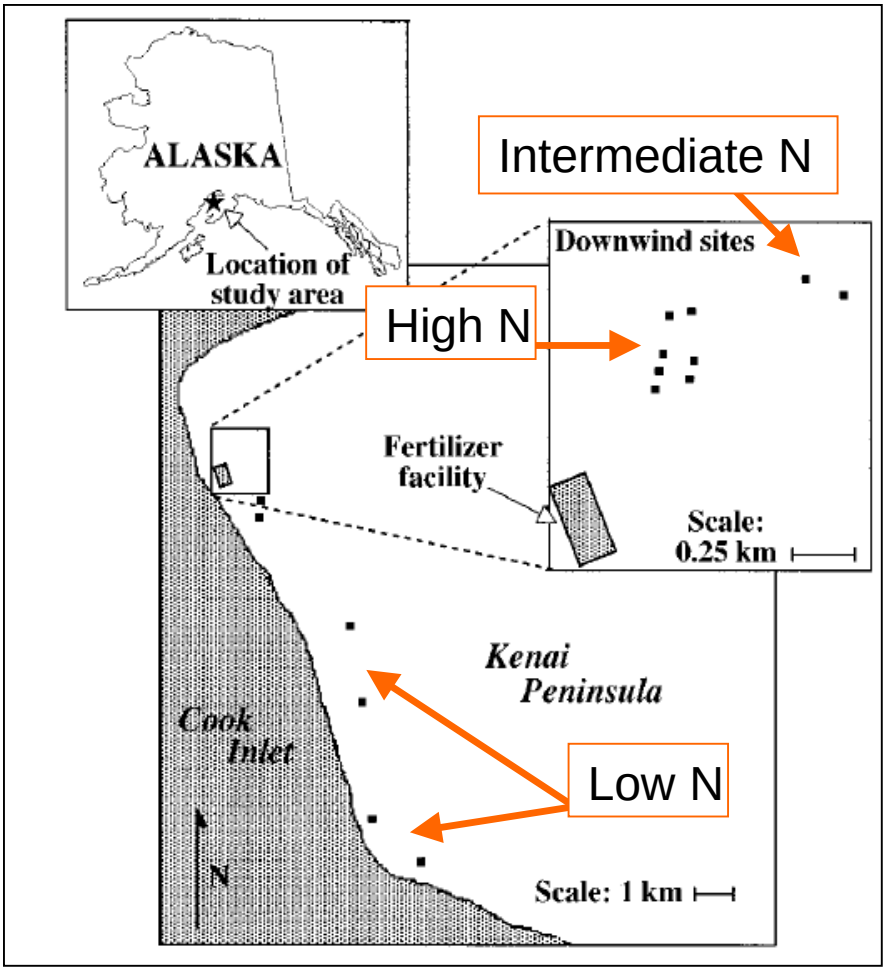


Figure 2 The study sites displaying defined low, intermediate, and high levels of N were located in a mature boreal forest on the Kenai Peninsula, in Alaska (modified from Lilleskov et al. 2001). An atmospheric ammonium deposition gradient exists near a fertilizer manufacturing facility with increased annual nitrogen deposition.

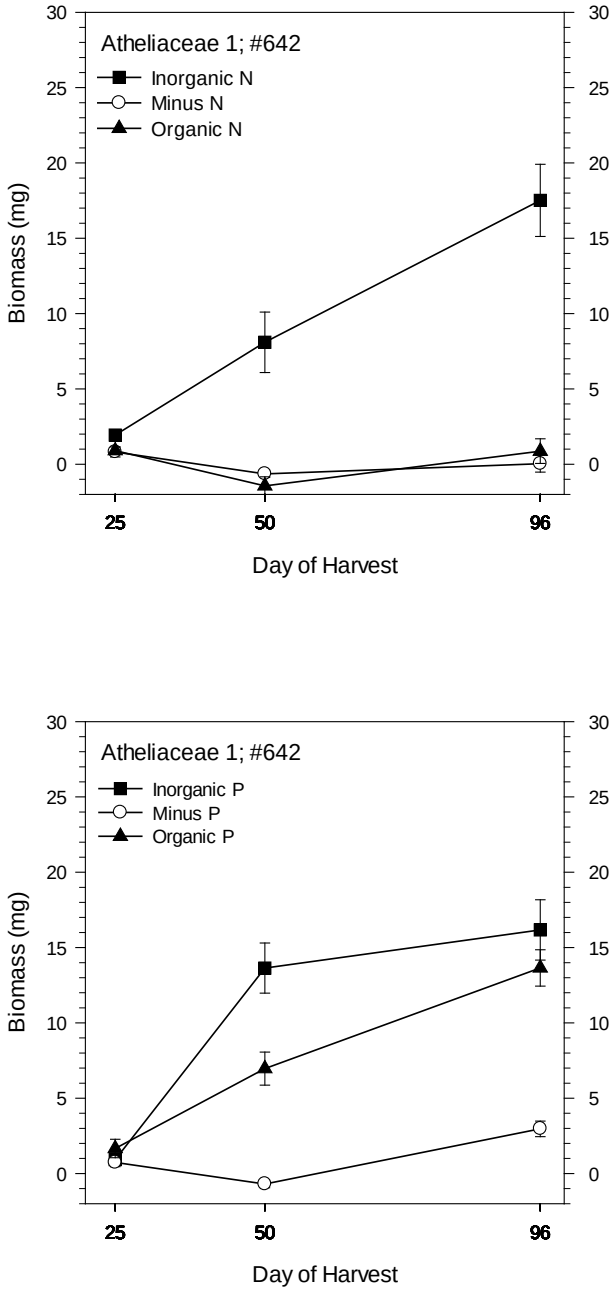


Figure 3 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles); and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

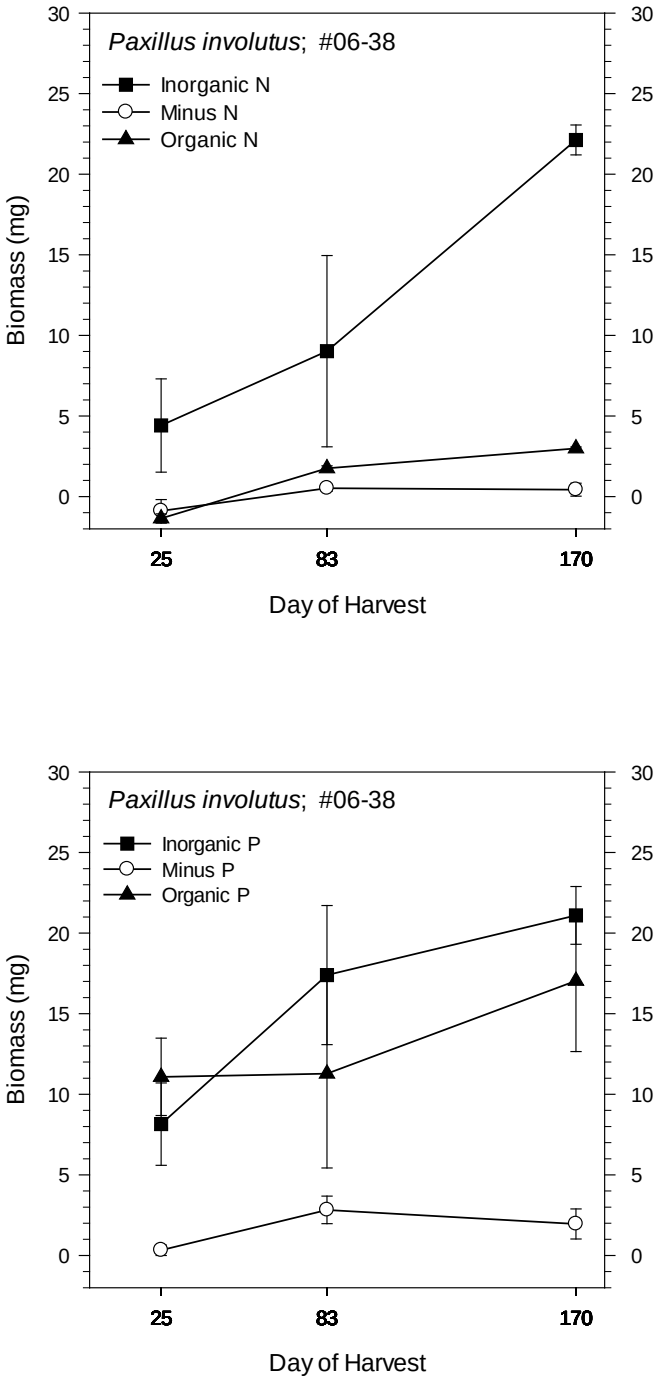


Figure 4 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

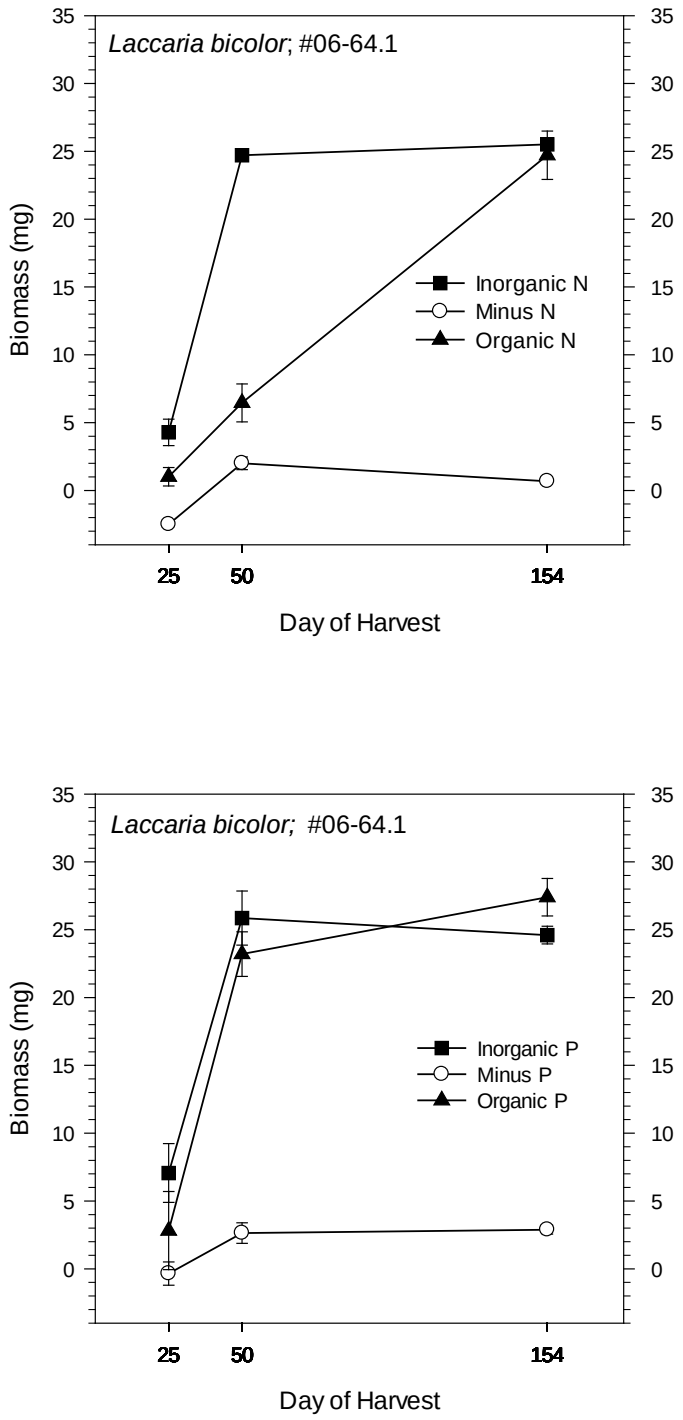


Figure 5 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

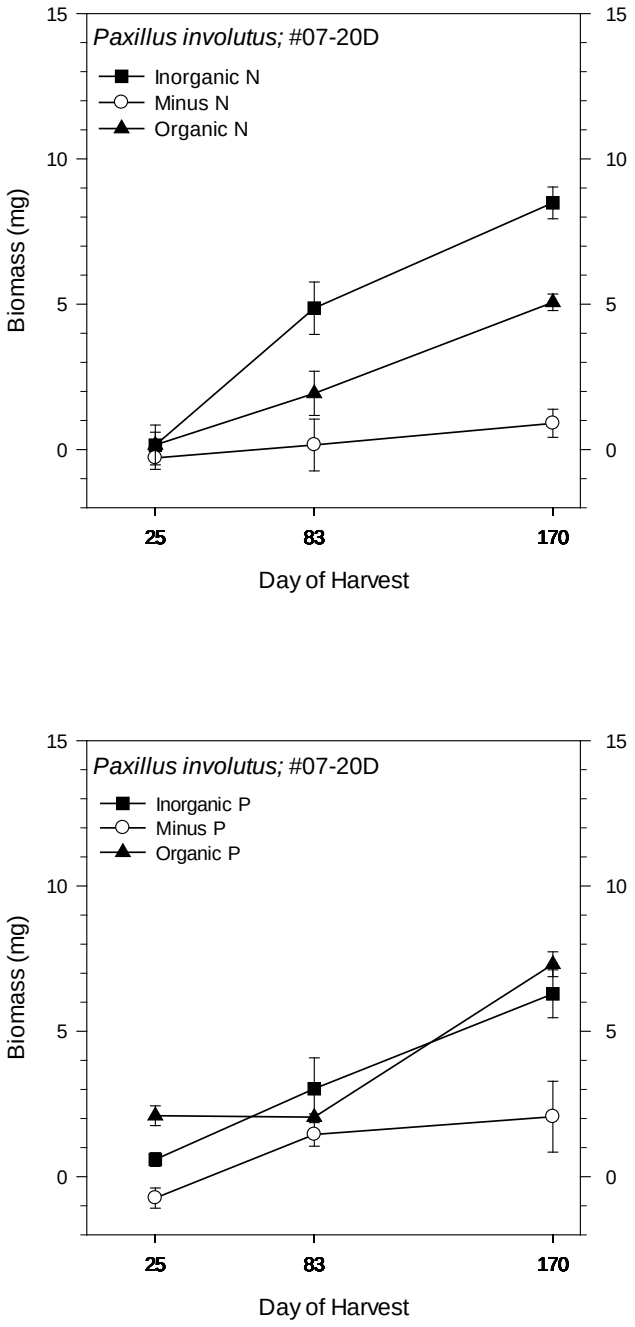
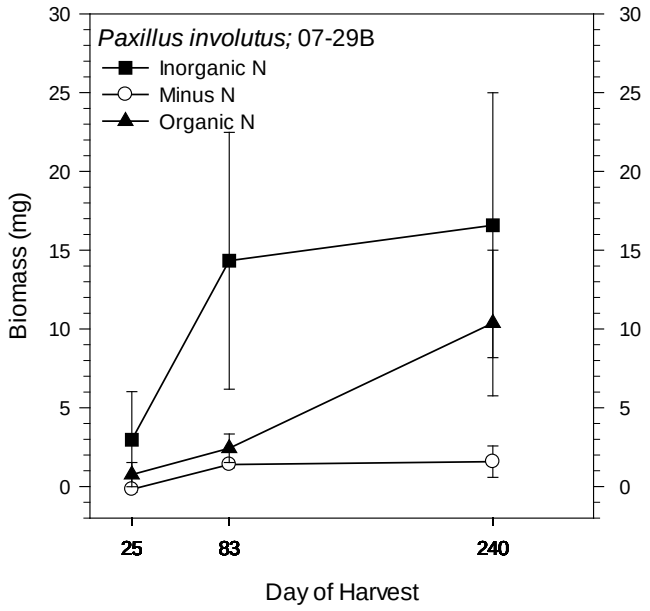


Figure 6 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3



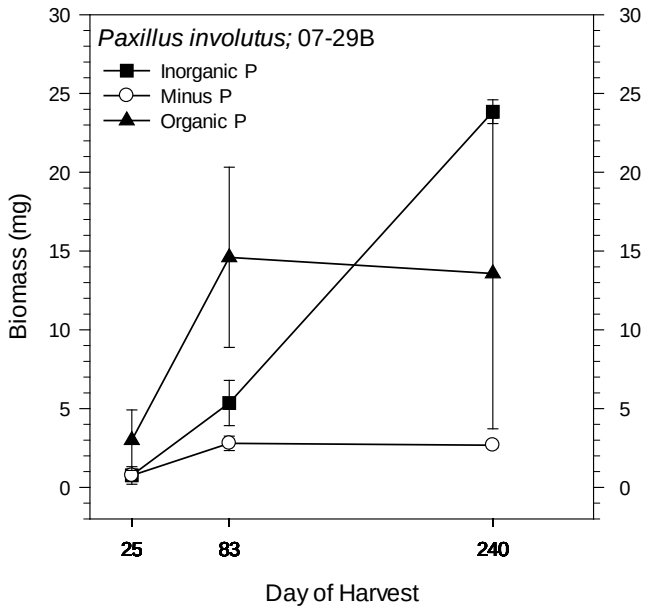


Figure 7 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

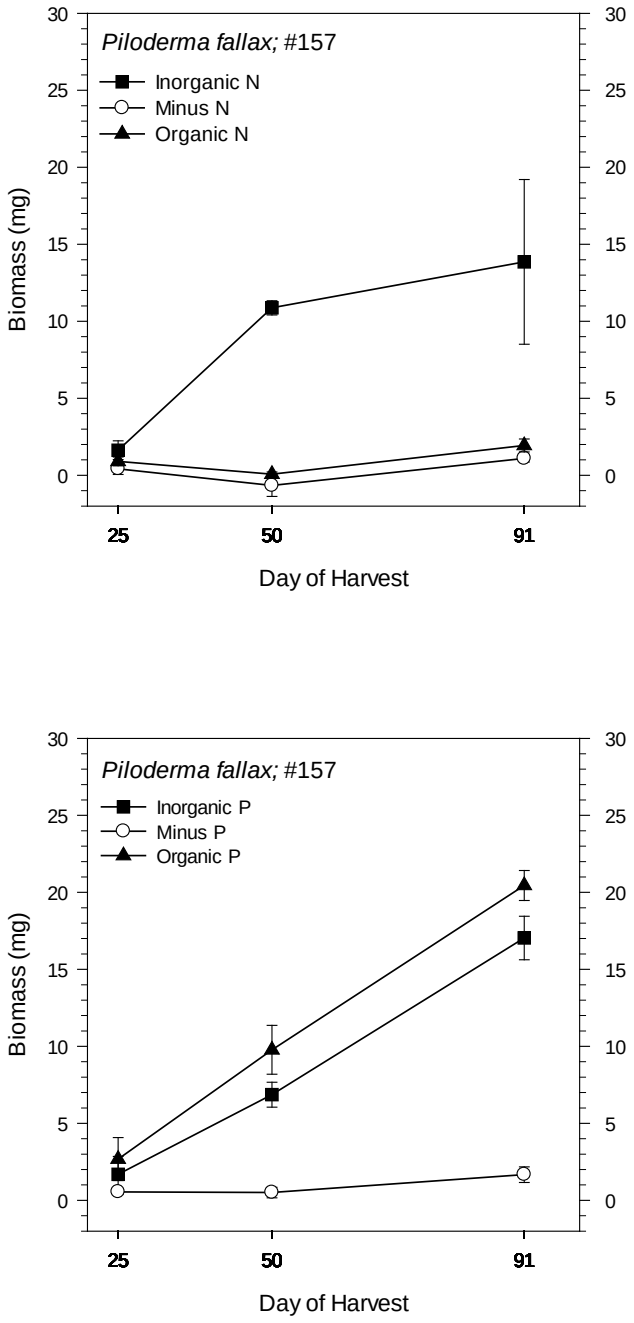


Figure 8 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

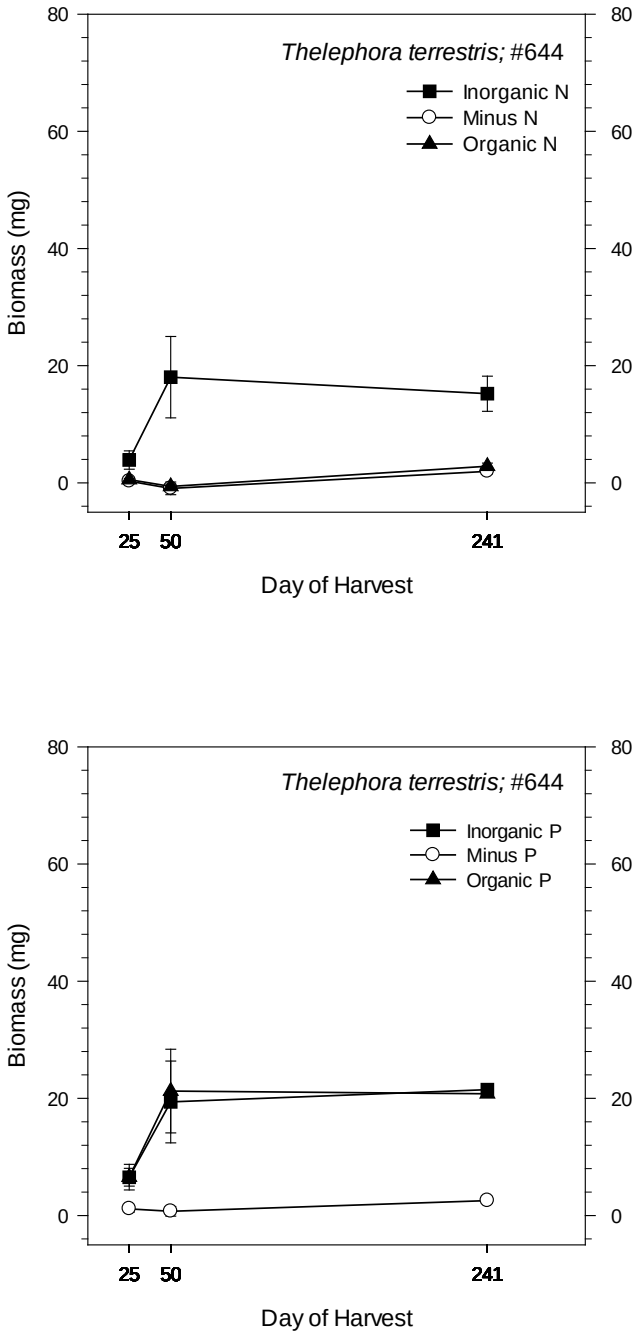


Figure 9 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

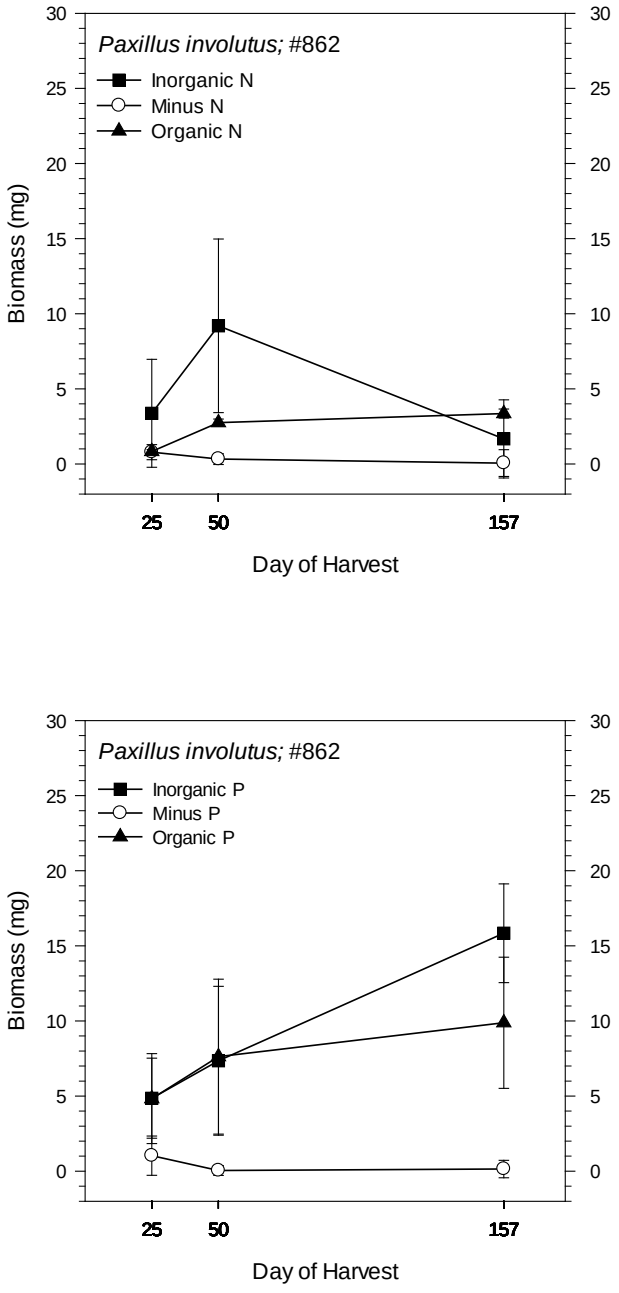


Figure 10 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

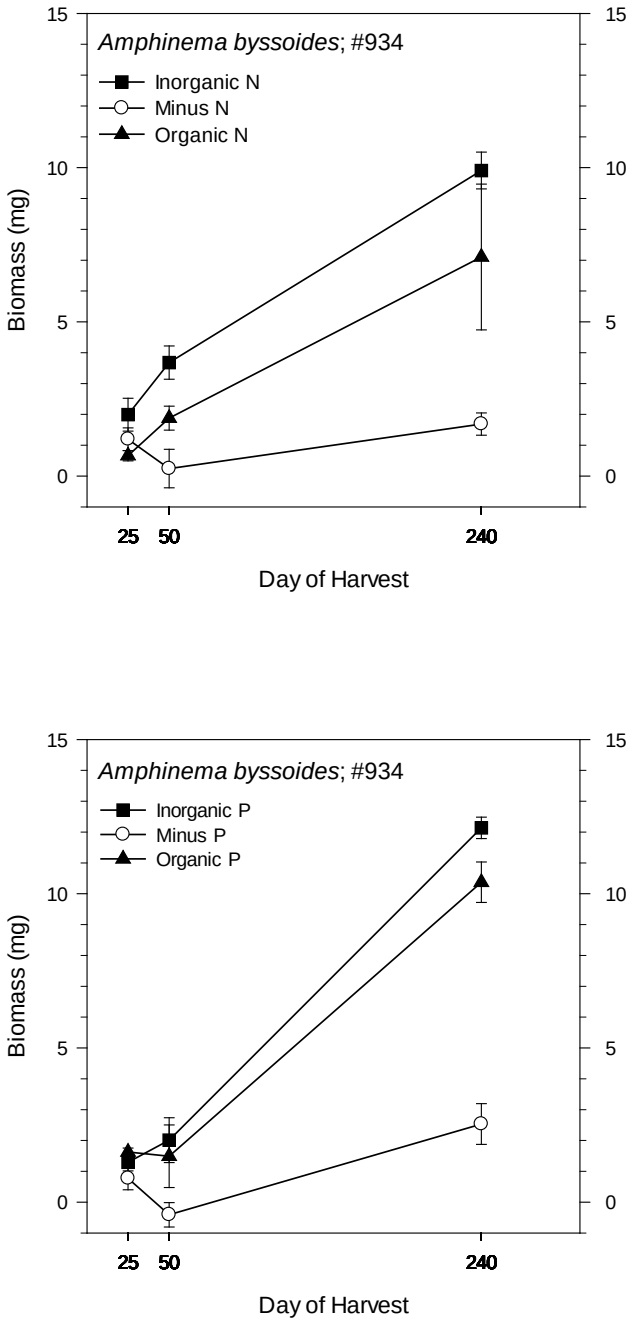
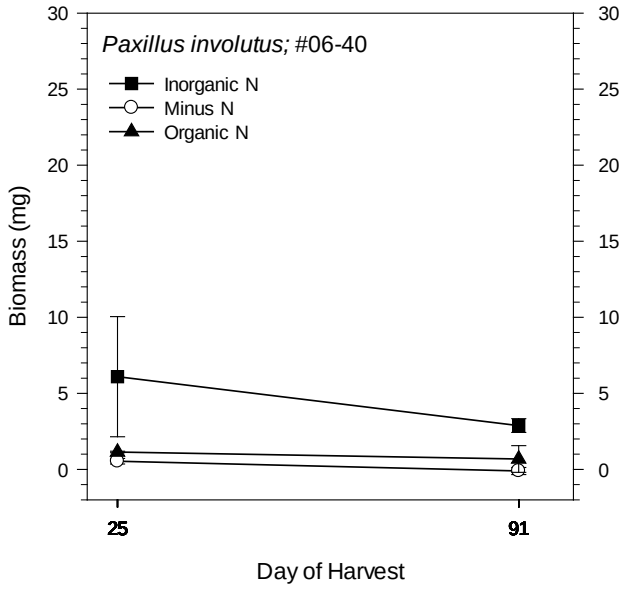


Figure 11 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3



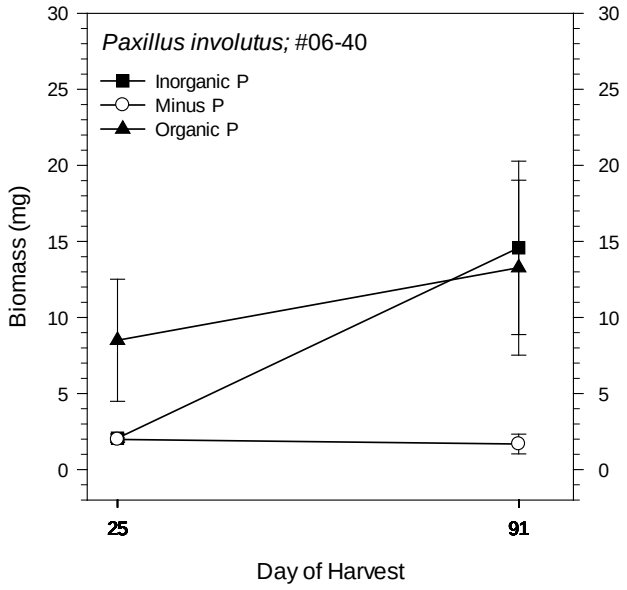
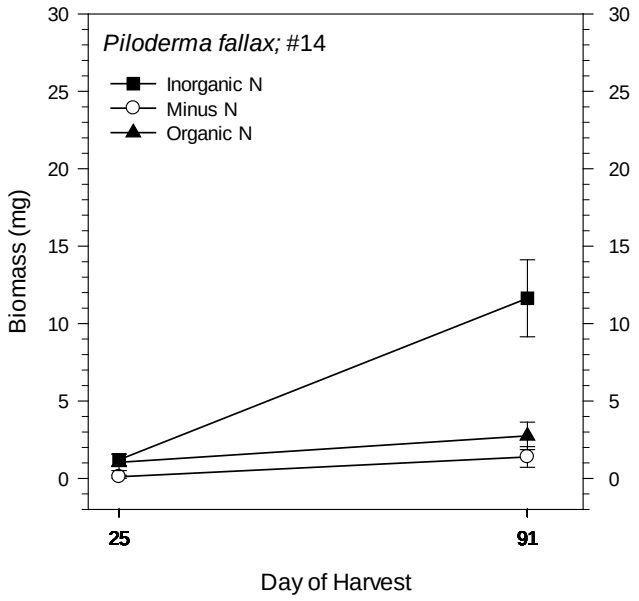


Figure 12 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3



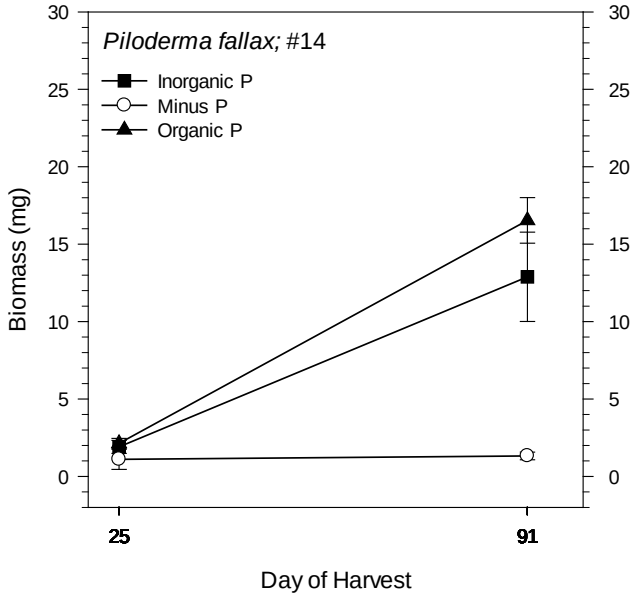


Figure 13 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

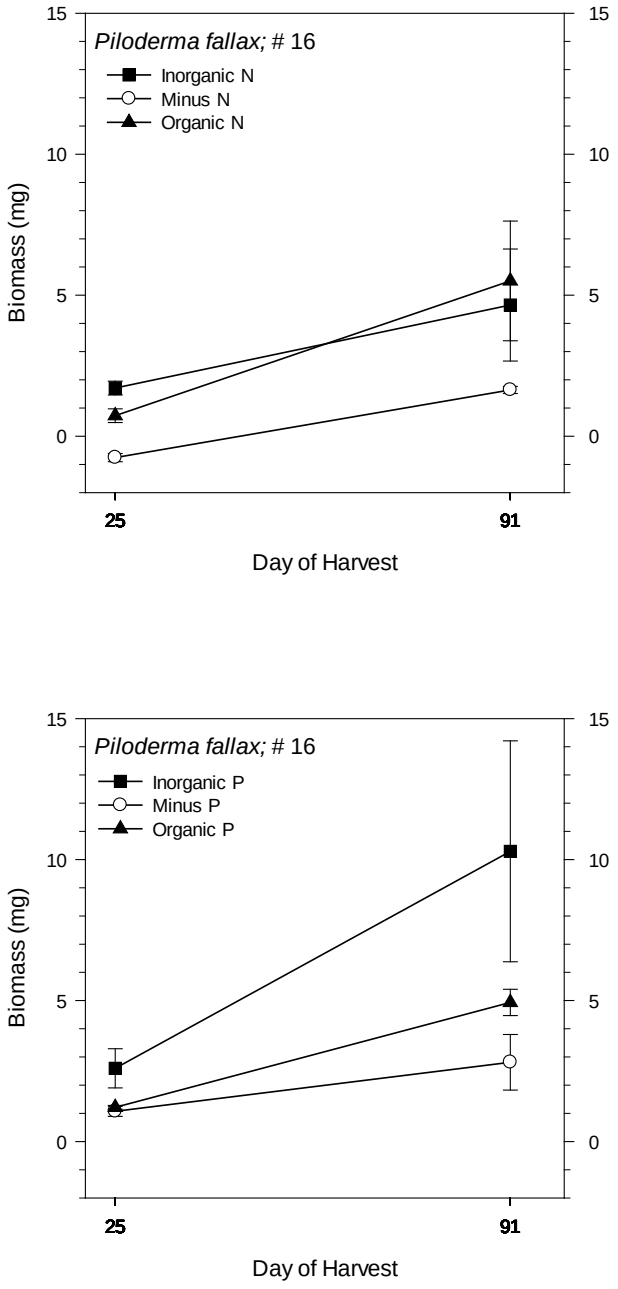
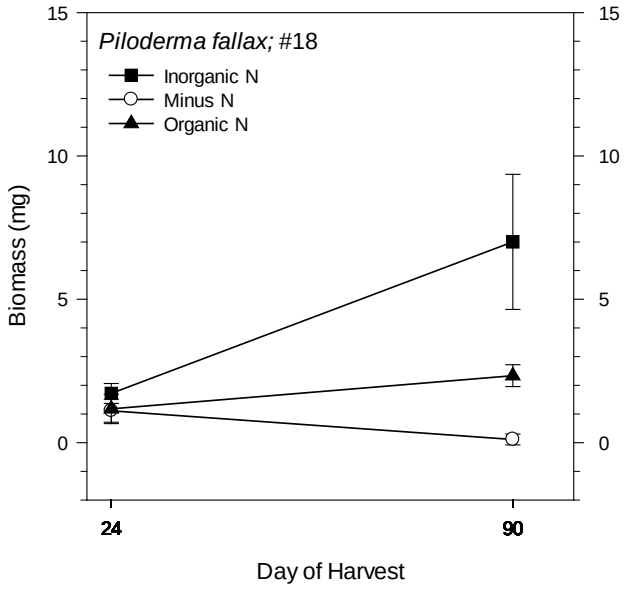


Figure 14 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3



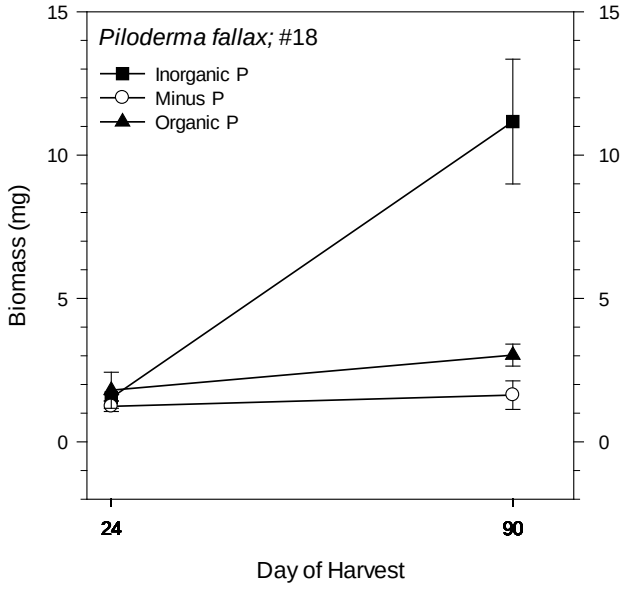


Figure 15 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

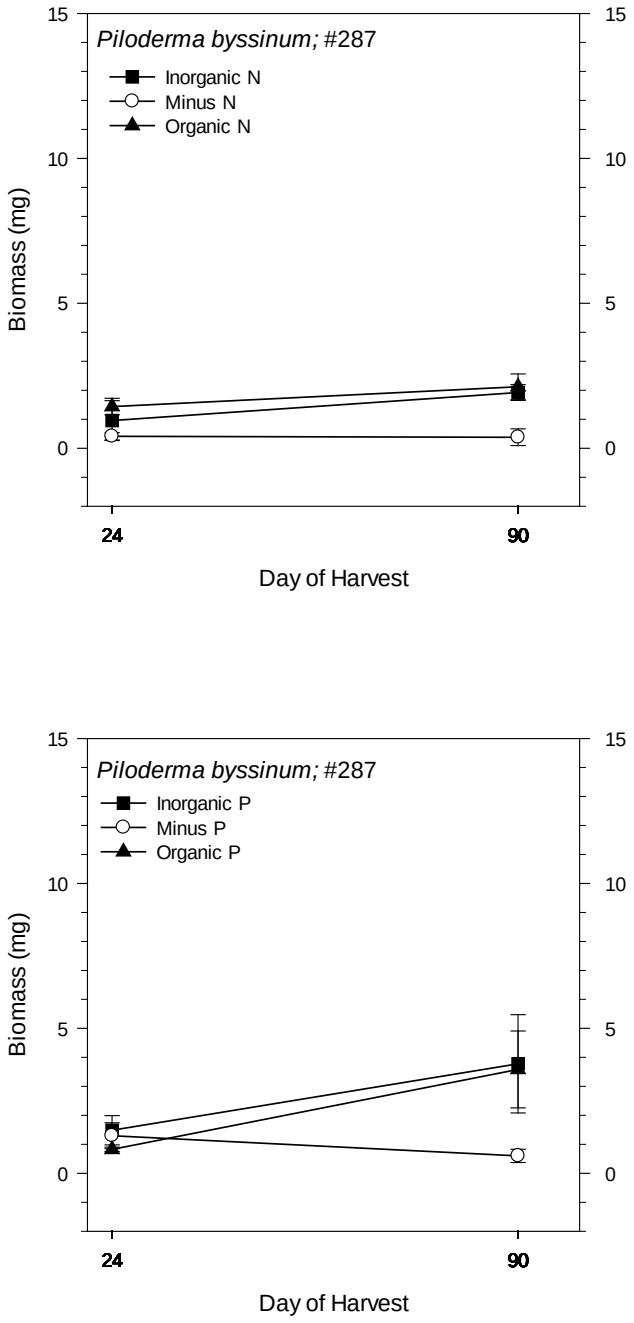


Figure 16 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

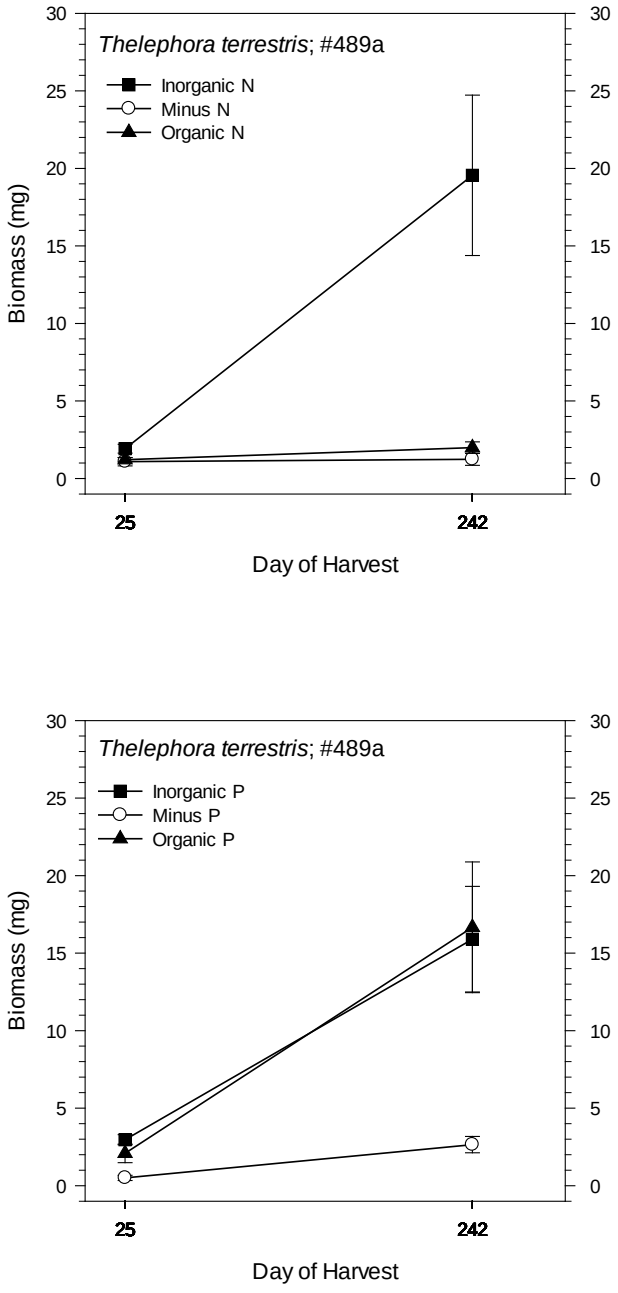
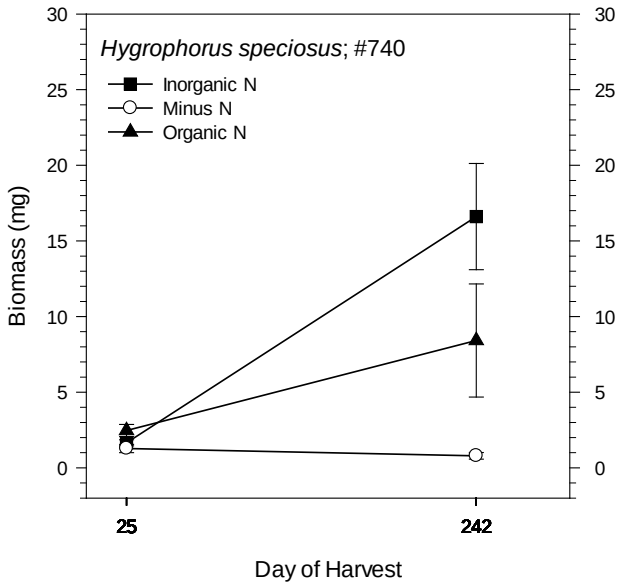


Figure 17 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3



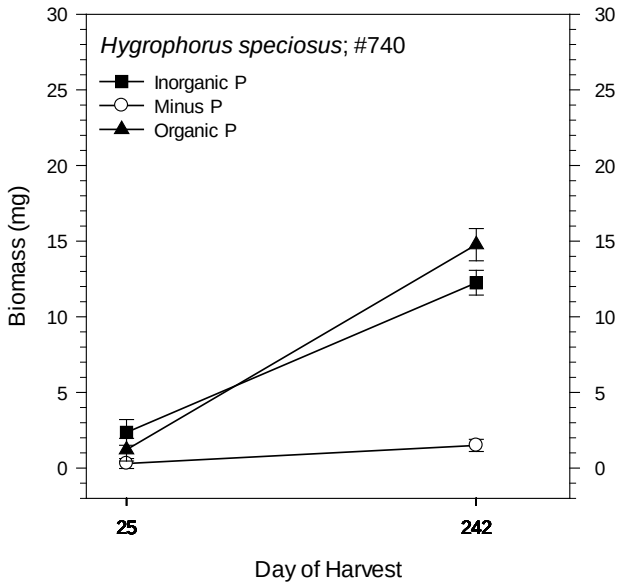


Figure 18 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

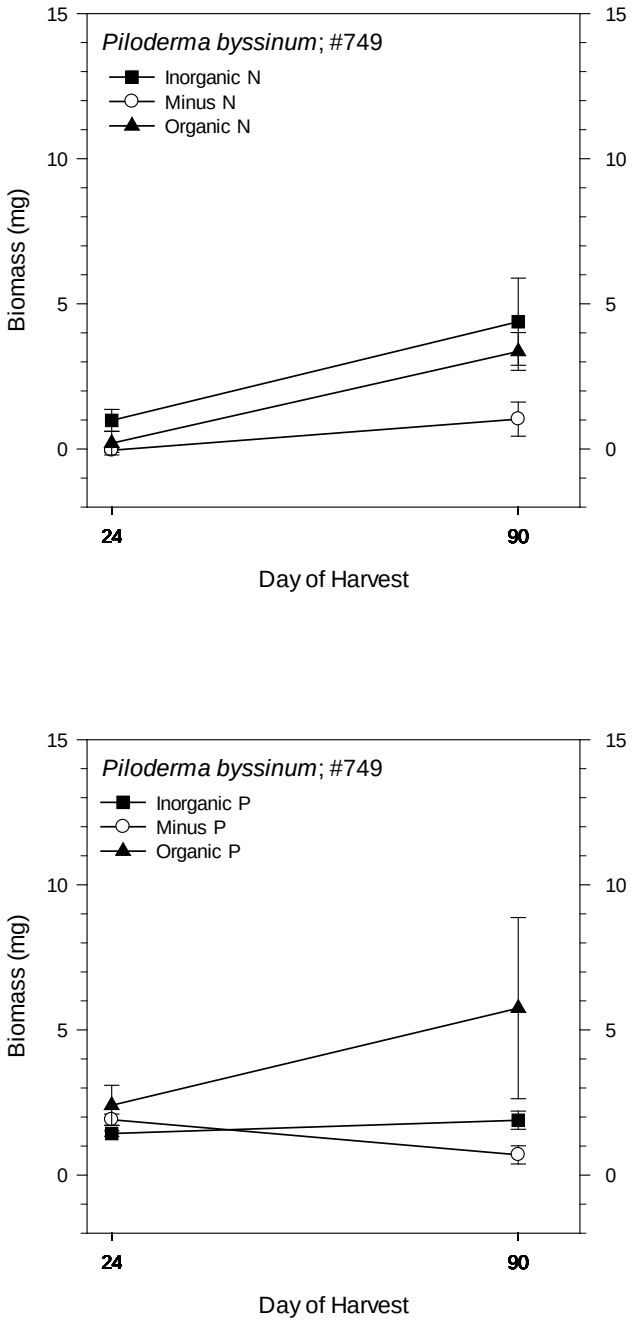


Figure 19 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

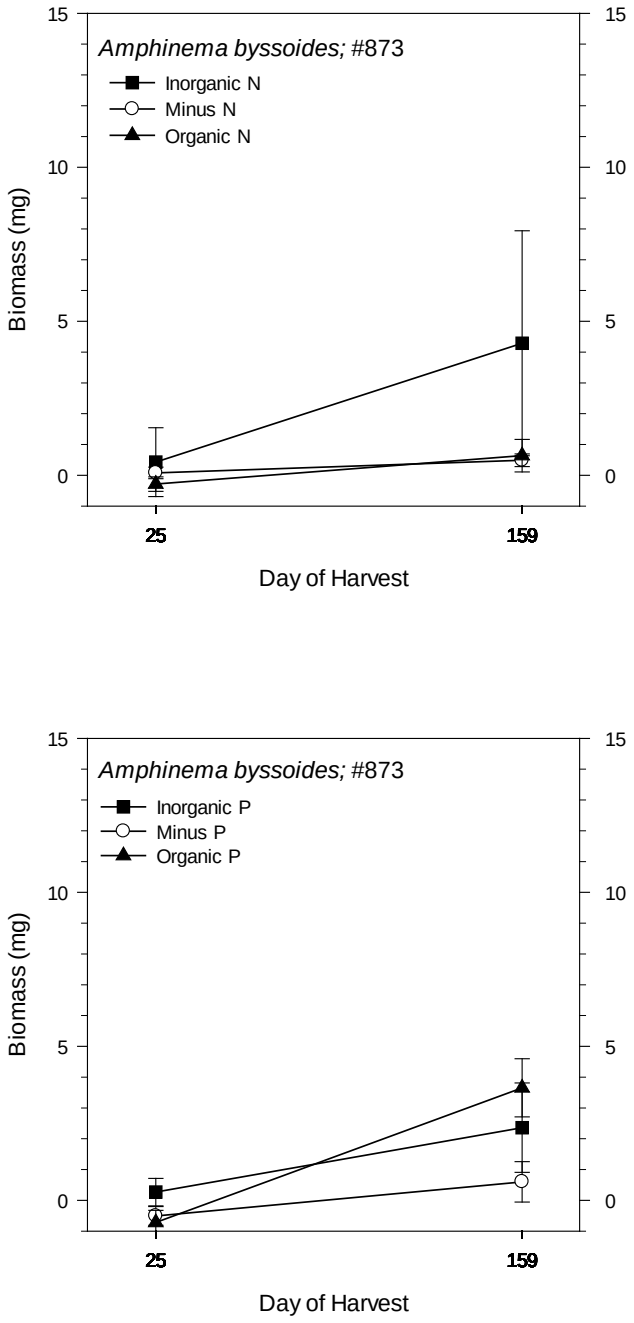


Figure 20 Pure culture growth rates (mean ± SE) on three different N sources (top graph): minus N control (open circles), NH_4Cl (closed squares), and bovine serum albumin (closed triangles) and on three different P sources (bottom graph): minus P control (open circles), KH_2PO_4 (closed squares), and phytic acid (closed triangles). n=3

Appendices

Appendix 1

Induction Medium Protocol: The basal medium was 10% strength MMN (Kottke et al. 1987) excluding malt extract and ammonium phosphate. Ammonium chloride (NH_4Cl) replaced the nitrogen source (N:P = 10) , citrate buffer was added to maintain the medium at pH 4.8, and glucose was added to remain as the sole carbon source.		
Component	**[1x]**	**units**
Macronutrients		
$CaCl_2$	5.0	mg/L
NaCl	2.5	mg/L
KH_2PO_4 (1/20th)	2.5	mg/L
NH_4Cl (N:P~10)	25	mg/L
$MgSO_4$ $7H_2O$	15	mg/L
Fe EDTA	5.0	mg/L
Organic nutrients		
Thiamine HCl	0.01	mg/L
Glucose	500.0	mg/L
Trace elements		
H_3BO_3	15.46	mg/L
$MnSO_4$*H_2O	8.45	mg/L
$ZnSO_4$*$7H_2O$	5.75	mg/L
$CuSO_4$*$5H_2O$	1.25	mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.0352	mg/L
Stock solution		
0.1M Citrate Buffer Stock	100.0	ml/L

Appendix 2

Medium Protocol for N Treatments: For organic N assays, the rudimentary medium for the experiment was MMN excluding malt extract. Citrate buffer was used across all treatments to maintain pH at 4.8.							
Component	**Minus N Treatments**		**NH4 N Treatments**		**BSA N Treatments**		
Macronutrients	[1x]	units	[1x]	units	[1x]	units	Sources
$CaCl_2*2H_2O$	66.2	mg/L	66.2	mg/L	66.2	mg/L	Lilleskov Lab MMN
NaCl	25	mg/L	25	mg/L	25	mg/L	Lilleskov Lab MMN
KH_2PO_4	500	mg/L	500	mg/L	500	mg/L	Lilleskov Lab MMN
$MgSO_4\,7H_2O$	150	mg/L	150	mg/L	150	mg/L	Lilleskov Lab MMN
Fe EDTA	5	mg/L	5	mg/L	5	mg/L	Lilleskov Lab MMN
Organic Nutrients							
Thiamine HCl	0.01	mg/L	0.01	mg/L	0.01	mg/L	Lilleskov Lab MMN
Glucose	5000	mg/L	5000	mg/L	5000	mg/L	Lilleskov Lab MMN
Trace elements							
H_3BO_3	15.46	mg/L	15.46	mg/L	15.46	mg/L	Kottke 1997
$MnSO_4*H_2O$	8.45	mg/L	8.45	mg/L	8.45	mg/L	Kottke 1997
$ZnSO_4*7H_2O$	5.75	mg/L	5.75	mg/L	5.75	mg/L	Kottke 1997
$CuSO_4*5H_2O$	1.25	mg/L	1.25	mg/L	1.25	mg/L	Kottke 1997
$Na_2MoO_4\cdot 2H_2O$	0.0352	mg/L	0.0352	mg/L	0.0352	mg/L	same [Mo] as Kottke 1997$(NH_4)6Mo_7O_{24}*4H_2O$
N Source 0.15g N/liter							
NH_4Cl			573	mg/L			Lilleskov et al. 2002
BSA					937.5	mg/L	Lilleskov et al. 2002 (16% N Finlay et al. 1992)
0.1M Citrate Buffer Stock	100	ml/L	100	ml/L	100	ml/L	Handbook of Biochemistry & Molecular Biology
	pH~4.8		N:P ~1.36				

Appendix 3

Medium Protocol for P Treatments: For organic P assays, the rudimentary medium for the experiment was MMN excluding malt extract. Citrate buffer was used across all treatments to maintain pH at 4.8.							
Component	**Minus P Treatments**		**PO4 P Treatment**		**C6H16CaO24P6 P Treatment**		
Macronutrients	[1x]	units	[1x]	units	[1x]	units	Sources
$CaCl_2*2H_2O$	66.2	mg/L	66.2	mg/L	66.2	mg/L	Lilleskov Lab MMN
NaCl	25.0	mg/L	25.0	mg/L	25.0	mg/L	Lilleskov Lab MMN
KCl	274.0	mg/L	274.0	mg/L	274.0	mg/L	same [K] as Lilleskov Lab MMN KH_2PO_4
NH_4Cl (N:P~10)	573.0	mg/L	573.0	mg/L	573.0	mg/L	Lilleskov Lab MMN
$MgSO_4$ 7H2O	150.0	mg/L	150.0	mg/L	150.0	mg/L	Lilleskov Lab MMN
Fe EDTA	5.0	mg/L	5.0	mg/L	5.0	mg/L	Lilleskov Lab MMN
Organic Nutrients							
Thiamine HCl	0.01	mg/L	0.01	mg/L	0.01	mg/L	Lilleskov Lab MMN
Glucose	5	mg/L	5	mg/L	5	mg/L	Lilleskov Lab MMN
Trace elements							
H_3BO_3	15.46	mg/L	15.46	mg/L	15.46	mg/L	Kottke 1997
$MnSO_4*H_2O$	8.45	mg/L	8.45	mg/L	8.45	mg/L	Kottke 1997
$ZnSO_4*7H_2O$	5.75	mg/L	5.75	mg/L	5.75	mg/L	Kottke 1997
$CuSO_4*5H_2O$	1.25	mg/L	1.25	mg/L	1.25	mg/L	Kottke 1997
$Na_2MoO_4 \cdot 2H_2O$	0.0352	mg/L	0.0352	mg/L	0.0352	mg/L	same [Mo] as Kottke 1997 $(NH_4)6Mo_7O_{24}*4H_2O$
P Source 0.00655g P/liter							
KH_2PO_4			65.9	mg/L			find reference for N:P in sporocarps
$C_6H_{16}K_2O_{24}P_6$					59.4	mg/L	find reference for N:P in sporocarps
0.1M Citrate Buffer Stock	100	ml/L	100	ml/L	100	ml/L	Handbook of Biochemistry & Molecular Biology
	pH~4.8		N:P=10				

Appendix 4

The name, formula, source with catalog code, and molecular weight of each chemical used in the experiment.				
Chemical Name	**Chemical Formula**	**Source**	**Catalog Code**	**Molecular Weight**
Calcium Chloride Dihydrate	$CaCl_2*2H_2O$	Fisher - ACROS	207780010	147.02
Sodium Chloride	NaCl	Sigma	S3014	58.44
Potassium Phosphate Monobasic	KH_2PO_4	Sigma	P-8416	136.1
Magnesium sulfate heptahydrate	$MgSO_4$ $7H_2O$	Sigma	M-2773	246.5
Ethylenediaminetetraacetic acid ferric sodium salt	Fe EDTA	Sigma	E-6760	367.1
Thiamine hydrochloride	Thiamine HCl	Sigma	T-1270	337.3
D-(+)-Glucose	Glucose	Fluka	49140	180.16
Boric Acid	H_3BO_3	VWR - BDH	BDH4118	61.83
Manganese(II) Sulfate Monohydrate	$MnSO_4*H_2O$	Fisher - ACROS	423915000	169.02
Zinc Sulfate, 7-Hydrate	$ZnSO_4*7H_2O$	Fisher - J.T. Baker	4382-01	287.56
Copper(II) Sulfate Pentahydrate	$CuSO_4*5H_2O$	Fisher - ACROS	423615000	249.68
Sodium molybdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	Sigma	331058	241.95
Ammonium chloride	NH_4Cl	Sigma	A4514	53.49
Albumin from bovine serum	BSA	Sigma	A3059	-
Potassium Chloride	KCl	Fisher	BP366	74.56
Citric Acid	$HOC(COOH)(CH_2COOH)_2$	Sigma	251275	192.12
Sodium Citrate	$HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$	Sigma	S1804	294.1
Phytic acid dipotassium salt	$C_6H_{16}K_2O_{24}P_6$	Sigma	P5681	736.2

Appendix 5

GROWTH EXERIMENT METHODS 09012009

Preparatory Work

- Supply List

    1. TEST TUBES
    2. TEST TUBE RACKS
    3. BSA
    4. INOSITOL HEXAPHOSPHATE
    5. FILTERS
    6. WIDE-MOUTH PINT BALL JARS

- Rate Cultures

    0 = no growth
    1 = growth barely present
    2 = some growth
    3 = definite growth
    4 = semi-substantial growth
    5 = substantial growth

- Decide on any cultures to expand on or eliminate
    - Tubes will not be tightly closed to optimize gas exchange and they will be taped with Parafilm
    - Mix at pH 4.8 and pipette into tubes and autoclave:
        1) MMN excluding NH_4Cl and malt extract with glucose and citrate buffer
        2) MMN excluding malt extract with glucose and citrate buffer
        3) MMN excluding NH_4Cl and malt extract with BSA added with glucose and citrate buffer
        4) MMN excluding KH_2PO_4 and malt extract with glucose and citrate buffer
        5) MMN excluding malt extract with glucose and citrate buffer
        6) MMN excluding KH_2PO_4 and malt extract with inositol hexaphosphate (Cumming 1993; Colpaert et.al. 1997; Hayes et.al. 2000) and glucose and citrate buffer
    - Select harvest dates (intervals) for each taxon

Process Date 1 (begin experiment):

- Blend liquid cultures in a Mason jar containing MMN liquid; let rest approximately two months
- Transfer by pipette each fungal isolate to nine test tubes of each growth medium and three to pre-weighed X mm filters for vacuum filtration and oven drying. This translates as each isolate must contain enough biomass to accommodate three filters and 54 test tubes allowing for three test tubes to be harvested from each growth treatment on three dates.
- Loosely screw down the test tube caps and parafilm.
- Store racks of test tubes in an incubator set at 15ºC
- Vacuum filter and wash with R.O. water three samples of each isolate in each treatment on a pre-weighed X mm filter; fold filter in half with forceps and place in labeled foil folders
- Freeze filters
- Freeze dry filters for 24 hours
- Place freeze dried filters into dessicators for 24 hours
- Weigh filters on a micro balance

Process Dates 2, 3, & 4:

- Randomly select three tubes of each isolate from each growth treatment
- Vacuum filter and wash with R.O. water three samples of each isolate in each treatment on a pre-weighed X mm filter; fold filter in half with forceps and place in labeled foil folders
- Freeze filters
- Freeze dry filters for 24 hours
- Place freeze dried filters into dessicators for 24 hours
- Weigh filters on a micro balance

Statistical Analysis in Minitab15

- Culture growth in the treatments compared to minus-N controls → ANOVA , DUNNETT
- Culture growth in the treatments compared to minus-P controls → ANOVA, DUNNETT