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Relationship between Arbuscular Mycorrhizal Fungal Development and Eupalitin Content in Bahiagrass Roots Grown in a Satsuma Mandarin Orchard

André Freire Cruz [1]*, Takaaki Ishii [1], Isao Matsumoto [2] and Kazuomi Kadoya [2]

[1] *Graduate School of Agriculture, Kyoto Prefectural University, Kyoto 606-8522*

[2] *Faculty of Agriculture, Ehime University, Matsuyama 790-8566*

Summary

An experiment was carried out in a satsuma mandarin orchard with a bahiagrass (*Paspalum notatum* Flügge.) covercrop to evaluate if the concentration of eupalitin, a growth stimulatory compound for arbuscular mycorrhizal (AM) fungi, is correlated with AM fungal development in soils. Root and soil samples were obtained monthly from the satsuma mandarin and bahiagrass to evaluate the AM infection in the roots and the number of AM spores in the soil. The root samples were kept in a freezer until analysed for eupalitin. Eupalitin was isolated and purified from bahiagrass root extracts by flash chromatography, gel filtration, and high pressure liquid chromatography (HPLC) and its concentration in each sample was determined based on a standard solution. The percentage of AM infection in both bahiagrass and satsuma mandarin roots was around 80% during the summer but decreased from October. The number of spores started to increase in March, attaining a maximum of around 800 in 25 g soil in June; the count subsequently decreased. Eupalitin concentration in bahiagrass roots was high from May to November, peaking in May and July. The concentration of eupalitin was significantly correlated with the AM infection in bahiagrass and the number of spores in soil, but not for the AM infection in satsuma mandarin. It suggests that there might be an interaction between AM fungal development and growth stimulatory compounds in soil.

Key Words: arbuscular mycorrhizal fungi, bahiagrass, citrus, eupalitin, sod culture.

Introduction

The sod culture system with bahiagrass plants in citrus orchards, a grass intercropped between citrus trees is well demonstrated to have some advantages; it improves soil texture, prevents soil erosion, contributes organic matter to the soil, and enhances the activity of AM fungi (Ishii et al., 2000b). Thus, soil management is an effective measure to introduce a sustained cultural system by using indigenous AM fungi to maintain these fungi in citrus orchards. *Bracchiaria decumbens* Benth. and *Vernonia* sp., also used as cover crops, have efficiency increased the AM fungus inoculum potential in soil (Schwob et al., 1999).

The use of bahiagrass in a sod culture system may further be justified because the roots release compounds which contain stimulatory substances for AM fungal activity; the AM fungi depend on living plant roots to be supplied by organic carbon (Kothari et al., 1990). The release of organic material occurs mainly as root exudates, which act as either signals or growth substrates for soil microorganisms (Barea et al., 2002). This growth activation can be defined as presymbiotic because it does not require physical root-fungus contact, but rather some diffusible specific roots exudates (Bago and Bécard, 2002). These authors also mentioned that even though the active root molecules seem to behave like chemical signals, the genetic evidence on the presymbiotic formation should be considered. In a sod culture system, however, the influence of the cover crop depends on whether the same AM fungus species will infect both plants, since there is some specificity between the AM fungi and the host plant (Sanders, 2003).

Ishii et al. (1997) isolated three kinds of growth stimulatory substances in the 25% methanol (MeOH) eluates of bahiagrass root extracts, which stimulated AM fungal development. One was identified as a flavonoid, eupalitin (3, 5-dihydroxy-6, 7-dimethoxy-4'-hydroxy flavone). According to these authors, this compound consists of phenyl groups, unsaturated carbonyl groups, aromatic methoxyl groups and hydroxyl groups, and the hydroxyl group in the position 3 of this flavonoid is essential to confer the stimulatory activity for AM hyphal growth. Among the three molecules isolated, eupalitin was the most efficient in promoting AM hyphal growth from germinating spores in vitro.

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* Corresponding author (E-mail: andre.feruz@usask.ca).

* Present address: Department of Soil Science, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, S7N5A8, Canada.

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In fact, bahiagrass root exudates contain several kinds of AM growth stimulatory compounds besides eupalitin. Ethylene (Ishii et al., 1996b), polyamines (Ishii et al., 2000a), alginate oligosaccharides (Ishii et al., 2000a), and other flavonoids (Buee et al., 2000) also have been demonstrated to stimulate the AM fungal activity. Likewise, flavonoid glycocides, such as narirutin, hesperidin (Aikawa et al., 2000) and rutin (Martins et al., 2000) have functioned as stimulators for AM infection. Recently, Nagahashi and Douds (1999) developed an in vitro assay, based on the branching response of *Gigaspora gigantea*, to test fractions of root exudates.

Eupalitin has been demonstrated in vitro to stimulate the hyphal growth of *Gigaspora ramisporosphora* (Ishii et al., 1997). However, the effect of eupalitin was not yet evaluated in vivo. The authors believe that by monitoring the fluctuation in spore number and infection and the concentration of eupalitin for one year, the behaviour of AM fungi in soils stimulated by eupalitin under natural conditions may be statistically predictable.

Materials and Methods

This experiment was conducted in a satsuma mandarin orchard with a bahiagrass covercrop at Ehime University, Japan. Monthly from March, 1998 to February, 1999, 4 soil samples were taken at 10cm depth from the satsuma mandarin orchard to evaluate the number of spores in 25 g soil, according to Ishii et al. (1996a). From those samples 2 sub-samples of 25 g were shaken in 70% of sucrose. The surface with floating spores was skimmed and transferred to petri dishes to observe the number of spores in stereoscope at 20x magnification. At the same time the root samples were obtained from satsuma mandarin and bahiagrass and fixed and preserved in FAA (formalin-acetic acid-ethyl alcohol) to determine the AM infection. These samples were washed and stained by the techniques of Phillips and Hayman (1970) and the percentage of AM infection in the roots was determined according to Ishii et al. (1996b).

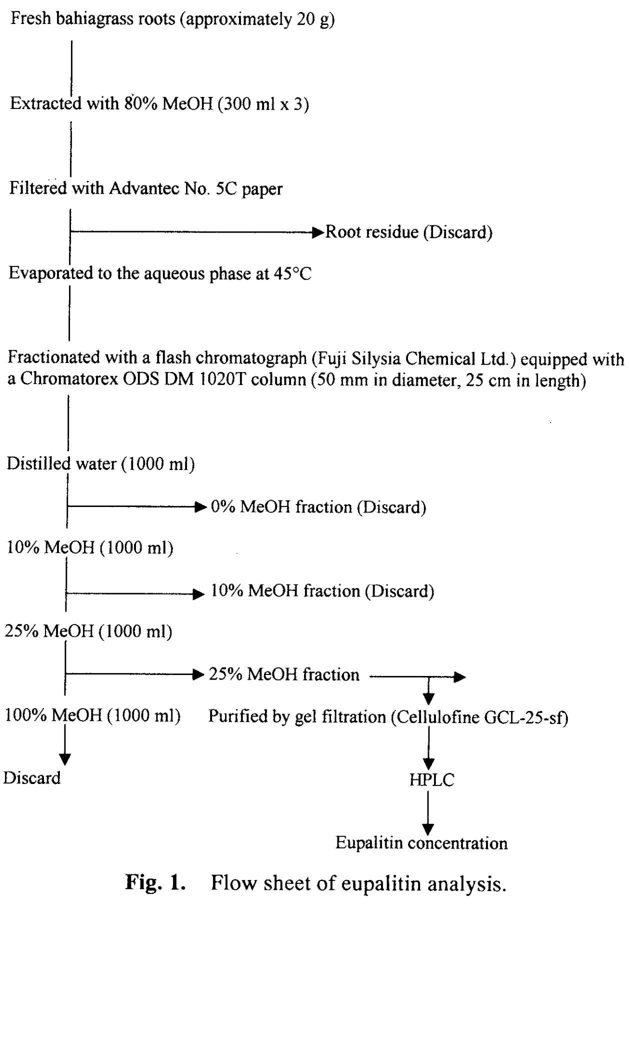

Fig. 1. Flow sheet of eupalitin analysis.

Bahiagrass roots (approximately 20 g) was sampled monthly and frozen until analysed for eupalitin. The isolation and purification of eupalitin were carried out according to Ishii et al. (1997) (Fig. 1). The 80% MeOH extracts of bahiagrass root were fractionated by flash chromatography. The 25% MeOH eluates were concentrated by an evaporator at 45℃ and the residue was purified by gel filtration using 10% of ethanol. Finally, eupalitin was obtained on a HPLC (ODS column: 10mm in diameter, 250 mm in length), by using a gradient solution of 50% MeOH. The concentration of eupalitin in each sample was determined using the standard solution of eupalitin already isolated and identified by Ishii et al. (1997).

Statistical analyses of data were done using correlation and general model procedures of the JMP software (SAS Institute Inc.). The relation between eupalitin concentration and the AM fungal development in the soil (% of AM infection and number of spores) was analysed statistically.

Results

The percentage of AM infection in bahiagrass and satsuma mandarin roots rapidly increased in May and July (Fig. 2). During the summer, the AM infection that

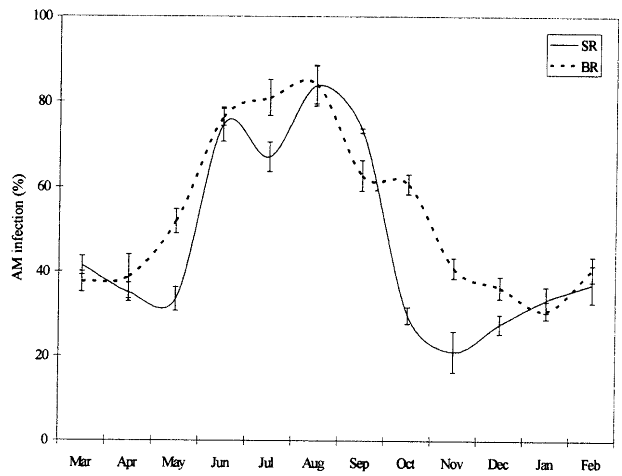

Fig. 2. Seasonal changes in the percentage of AM infection of bahiagrass roots (BR) and satsuma mandarin roots (SR) in a satsuma mandarin orchard sodded with bahiagrass. Vertical bars indicate standard error (SE) (n=4).

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peaked around 80%, decreased after September. The rate of decrease was more rapid in satsuma mandarin than in bahiagrass roots. The AM infection was higher in bahiagrass than in satsuma mandarin from April to July and from October to January

The number of spores started to increase in March, and reached a maximum of around 800 in 25 g soil in June, and then decreased to around 300 until August; a little increase occurred in September. From October to February spore count was stable between 200 and 300 (Fig. 3). The number of spores from May to July was twice that in February.

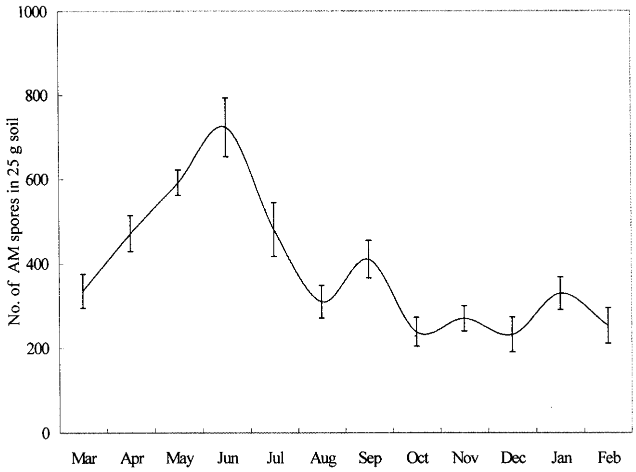

Fig. 3. Seasonal changes in the number of AM spores in the soil of a satsuma mandarin orchard sodded with bahiagrass. Vertical bars indicate SE (n=4).

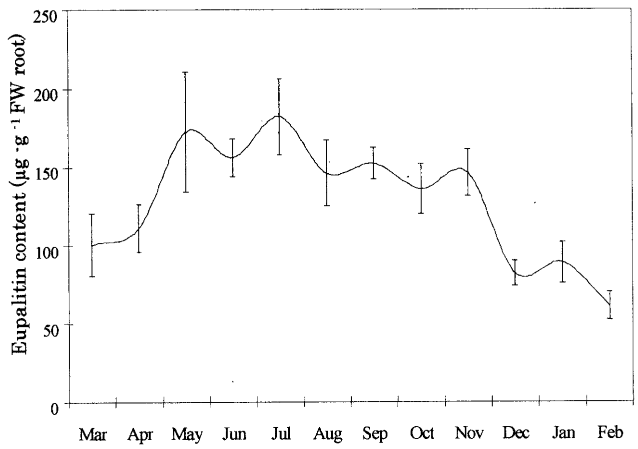

Fig. 4. Seasonal changes in the eupalitin concentration in bahiagrass roots in a satsuma mandarin orchard sodded with bahiagrass. Vertical bars indicate SE (n=4).

The concentration of eupalitin in bahiagrass roots was high from May to November, in particular, in May and July, the growing season. During this period, the concentration was over twice that in February (Fig. 4). Hence, the relationship between eupalitin and the percentage of infection and number of spores exhibited a good fit during the growing season, even though their peaks did not always coincide.

Eupalitin concentration was significantly correlated with the percentage of infection in bahiagrass (R^2=0.54, P<0.0064), and the number of spores (R^2=0.34, P<0.047), but not with the infection in satsuma mandarin (R^2=0.22, P< 0.11) (Table 1).

Discussion

The AM infection was considerably high i.e. around 30% or more and number of spores too, which indicates the bahiagrass sod culture system as an effective soil management practice for the propagation and establishment of AM fungi in citrus orchards. This is especially true during the summer when increased AM infection and spores could improve mineral absorption by roots. Previous works have documented the influence of some weeds on AM formation in citrus orchards (Ishii et al., 1989). Also, seasonal variations in AM infection and spores were evaluated by other researches who took into account the host plant and environmental stresses (Carvalho et al., 2001, Lugo et al., 2003). Previous studies by Ishii et al. (1996a) on seasonal spore number in citrus orchards sodded with bahiagrass showed that the spore populations and AM infection increased after the growing season. However, Brundrett and Abbott (1994) reported that there were no substantial seasonal fluctuations in mycorrhizal formation in clover roots and that the capacity for root colonization by AM fungus propagules was maintained throughout a year. Similar results were found by Schwob et al. (1999) in a tropical rubber tree plantation where no marked seasonal variations in mycorrhizal formation occurred.

The variation in AM spores and infection in the present study was generally similar to variation of the eupalitin concentration, but not linearly related (Table 1); a significant correlation occurred only for the AM infection in bahiagrass and number of spores. Perhaps, the bahiagrass roots are induced to exude a larger

Table 1. Regression models obtained by plotting the eupalitin concentration (Eup), versus AM fungal development (% of infection; No. of spores).

	Equation	R^2	RMSE	Prob.>F	n
InfBG x Eup	y = 6.96 + 0.37x	0.54	13.45	0.0064	12
InfSM x Eup	y = 11.73 + 0.27x	0.22	19.95	0.11	12
NSpo x Eup	y = 87.17 + 2.34x	0.34	131.37	0.047	12

InfBG: % of infection in bahiagrass roots, InfSM: % of infection in satsuma mandarin roots, NSpo: Number of spores in the soil, RMSE: Root mean square error, n: Number of observations.

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quantity of compounds containing AM growth stimulators, such as eupalitin, to activate the AM fungi in soils during the growing season. Since the peak of eupalitin was before the maximum of number of spores, the releasing of growth stimulatory compounds in the soil might be before the increasing of AM fungus propagules. However, this contribution may not be the main factor, because other growth stimulatory compounds (Martins et al., 2000) and molecular signals (Sanders, 2003) are also involved in the AM infection and spore formation in soils.

That AM infection reached a maximum in summer and decreased in spring and in fall in satsuma mandarin roots may indicate that the roots grow in spring, their growth is inhibited during the summer when aerial growth flourishes, and roots resume growth when shoot growth ceases in the fall. This pattern of cyclic growth could explain the lack of relationship between eupalitin and root colonization by AM fungi in satsuma mandarin. Therefore, the non-significant correlation between eupalitin and root colonization in satsuma mandarin does not mean that bahiagrass sod culture system in citrus orchards has no influence in citrus orchards.

The bahiagrass root exudates contain several chemical compounds, including stimulators and inhibitors of AM fungal activity. Eupalitin acts as a general stimulant for AM fungi, but the process of sporulation and infection are also triggered by other signals. However, it seems to contribute as a stimulant in the exudates when they are released to the soil. Eupalitin was demonstrated to promote growth of hyphae in vitro (Ishii et al., 1997) and in an experiment under greenhouse (unpublished data) the 25% MeOH eluates of bahiagrass root extracts, which contain eupalitin, increased AM infection in trifoliate orange rootstocks as compared to other fractions. Cruz et al. (2002) also reported that this fraction stimulated the growth of AM hyphae in the soil even in the absent of the host plant. These in vitro and in vivo results offer evidence about the contribution of eupalitin for AM fungal activity in soils, even though the greenhouse experiments were conducted with a crude extract and not pure eupalitin.

Our data indicate that from early spring to summer, the seasonal variation of AM infection in bahiagrass and number of spores had significant relations with the one of eupalitin concentration in bahiagrass roots, although these results are statistically weak. Currently, we have evidence that bahiagrass roots would release several compounds in the soil system, including the AM growth stimulants, such as eupalitin that activates AM fungi in soils. Further studies are necessary to evaluate whether the AM growth stimulatory substances extracted from the roots would influence AM formation in the ecosystem of citrus orchards.

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Literature Cited

Aikawa, J., T. Ishii, Kuramoto, M. and K. Kadoya. 2000. Growth stimulants for vesicular-arbuscular mycorrhizal fungi in satsuma mandarin pomace. J. Japan. Soc. Hort. Sci. 69: 385-389.

Bago, B. and G. Bécard. 2002. Bases of the obligate biotrophy of arbuscular mycorrhizal fungi. p. 33-48. In: S. Gianinazzi, H. Schuepp, J. M. Barea and K. Haselwandter (eds.). Mycorrhizal technology in agriculture: From genes to bioproducts. Birkhauser verlag ag. Basel, Boston, Berlin.

Barea, J. M., M. Gryndler, P. Lemanceau, H. Schuepp and R. Azcon. 2002. The rhizosphere of mycorrhizal plants. fungi. p 1-18. In: S. Gianinazzi, H. Schuepp, J. M. Barea and K. Haselwandter (eds.). Mycorrhizal technology in agriculture: From genes to bioproducts. Birkhauser verlag ag. Basel, Boston, Berlin.

Brundrett, M. C. and L. K. Abbott. 1994. Mycorrhizal fungus propagules in the Jarrah forest-I. Seasonal study of inoculum levels. New Phytol. 127: 539-546.

Buee, M., M. Rossignol, A. Jauneau, R. Ranjeva and G. Becard. 2000. The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Molec. Plant-Microbe Interac. 13: 693-698.

Carvalho, L. M., I. Caçador and M. A. Martins-Loução. 2001. Temporal and spatial variation of arbuscular mycorrhizas in salt marsh plants of the Tagus estuary (Portugal). Mycorrhiza 11: 303-309.

Cruz, A. F., T. Ishii, I. Matsumoto and K. Kadoya. 2002. Network establishment of vesicular-arbuscular mycorrhizal hyphae in the rhizospheres between trifoliate orange and some plants. J. Japan. Soc. Hort. Sci. 71: 19-25.

Ishii, T., J. Aikawa, S. Kirino, H. Kitabayashi, I. Matsumoto and K. Kadoya. 2000a. Effects of alginate oligosaccharide and polyamines on hyphal growth of vesicular-arbuscular mycorrhizal fungi and their infectivity of citrus roots. Proc. Int. Soc. Citriculture: 1030-1032.

Ishii, T., S. Kirino and K. Kadoya. 2000b. Sustainable citriculture using vesicular-arbuscular mycorrhizal fungi: Introduction of new soil management techniques. Proc. Int. Soc. Citriculture: 1026-1029.

Ishii, T., A. Narutaki, K. Sawada, J. Aikawa, I. Matsumoto and K. Kadoya. 1997. Growth stimulatory substances for vesicular-arbuscular mycorrhizal fungi in Bahia grass (*Paspalum notatum* Flügge.) roots. Plant and Soil 196: 301-304.

Ishii, T., Y. H. Shrestha and K. Kadoya. 1996a. Effect of sod culture system of Bahia grass (*Paspalum notatum* Flügge.) on vesicular-arbuscular mycorrhizal forma-

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tion of satsuma mandarin trees. Proc. Int. Soc. Citriculture 2: 822–824.

Ishii, T., Y. H. Shrestha, I. Matsumoto and K. Kadoya. 1996b. Effect of ethylene on the growth of vesicular-arbuscular mycorrhizal fungi and on the mycorrhizal formation of trifoliate orange roots. J. Japan. Soc. Hort. Sci. 65: 525–529.

Ishii, T., K. Tasumi and K. Kadoya. 1989. Distribution and ecological aspects of vesicular arbuscular mycorrhizal fungi in citrus orchards. Mem. Coll. Agr., Ehime Univ. 34: 65–71.

Kothari S. K., H. Marschner and E. George. 1990. Effect of VA mycorrhizal fungi and rhizosphere microorganisms on root and shoot morphology, growth and water relations in maize. New Phytol. 116: 303–311.

Lugo, M. A., M. E. Gonzalez-Maza and M. N. Cabello. 2003. Arbuscular mycorrhizal fungi in a mountain grassland II: Seasonal variation of colonization studied, along with its relation to grazing and metabolic host type. Mycologia 95: 407–415.

Martins, M. A., G. F. Goncalves and A. C. F. Soares. 2000. Effect of arbucular mycorrhizal fungi associated to phenolics compounds on growth of papaya plants. Pesq. Agropec. Bras. 35: 1465–1471 (In Portuguese).

Nagahashi, G. and D. D. Douds. 1999. Rapid and sensitive bioassay to study signals between root exudates and arbuscular mycorrhizal fungi. Biotech. Techn. 13: 893–897.

Phillips, J. M. and D. S. Hayman. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55: 158–161.

Sanders, I. R. 2003. Preference, specificity and cheating in the arbuscular mycorrhizal symbiosis. Trends in Plant Sci. 8: 143–145.

Schwob, I., M. Ducher and A. Coudret. 1999. Effect of climatic factors on native arbuscular mycorrhizae and *Meloidogyne exigua* in a Brazilian rubber tree (*Hevea brasiliensis*) plantation. Plant Path. 48: 19–25.

ウンシュウミカン園におけるバヒアグラス根内のユーパリチン含量とアーバスキュラー菌根菌の発達との関係

アンドレ フェレイレ クルス[1]*・石井孝昭[1]・松本 勲[2]・門屋一臣[2]

[1]京都府立大学大学院農学研究科 606-8522 京都市左京区下鴨半木町

[2]愛媛大学農学部 790-8566 松山市樽味

摘 要

バヒアグラス草生のウンシュウミカン園において，アーバスキュラー菌根(AM)菌生長促進物質の一つであるユーパリチン含量と菌根菌の発達との関連について調査した．菌根形成や土壌中のAM菌の胞子数を調査するため，ウンシュウミカンおよびバヒアグラスの根，ならびに土壌を1か月に1回採取した．またユーパリチン含量を分析するため，バヒアグラス根も毎月採取し，冷凍庫に保存した．ユーパリチンはフラッシュクロマトグラフィー，ゲルろ過および高速液体クロマトグラフィーで単離・精製し，ユーパリチン標準液を用いて定量した．その結果，バヒアグラスおよびウンシュウミカンの菌根感染率は夏季において最も高く(約80%)，10月から感染率は低下した．土壌中の胞子数は3月から増加し始め，6月に最大胞子数(土壌25 g中約800個)となった後，低下した．バヒアグラス根内のユーパリチン含量は5月から11月の期間において高く，特に5月および7月の高まりは顕著であった．根内のユーパリチン含量とウンシュウミカンの菌根感染率との間には明らかな相関がみられなかったが，バヒアグラスの菌根感染率や土壌中の胞子数との間には有意な相関が認められた．このことは，土壌におけるAM菌生長促進物質含量と菌根菌の発達との間には密接な相互関係が存在することを示唆している．

* 現在: Department of Soil Science, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, S7N5A8, Canada.

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