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Research Note

Mycorrhizal colonisation of highbush blueberry and its native relatives in central Finland

Anne Kasurinen, Toini Holopainen
Department of Ecology and Environmental Science, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland, e-mail: anne.kasurinen@uku.fi

Seija Anttonen
The Finnish Forest Research Institute, Suonenjoki Research Station, FIN-77600 Suonenjoki, Finland

Transmission electromicroscopy, trypan blue staining in combination with stereomicroscope analysis and biochemical ergosterol assay were used to study the mycorrhizal symbionts in wild bilberry (*Vaccinium myrtillus*), bog whortleberry (*Vaccinium uliginosum*) and highbush blueberry (*Vaccinium corymbosum*) roots. TEM-analysis showed that in all species ericoid mycorrhizas formed hyphae coil inside the epidermal root cells. In stereomicroscopic viewing the highest mycorrhizal colonisation was observed in the roots of wild bilberries (51%), whereas according to the ergosterol assay the highest total fungal biomass of roots was found in bog whortleberries (209 $\mu g\ g^{-1}$ of root d. wt). Both ergosterol and microscopical method showed that the mycorrhizal associations in blueberry cultivars and their wild relatives growing on natural soil medium are frequent, although ericoid mycorrhiza formation of highbush blueberries was somewhat weaker than that of wild bilberries and bog whortleberries.

Key words: ergosterol, ericoid mycorrhiza, highbush blueberry, wild bilberry, bog whortleberry, *Vaccinium* sp.

Introduction

The first highbush blueberry cultivars were hybridized from naturally occurring *Vaccinium australe*, *V. corymbosum* and *V. angustifolium* blueberry species in North America at the beginning of this century. Since then several new blueberry hybrids have been developed for commercial as well as home garden use (Luby et al. 1986) and commercial cultivation of highbush blueberries has spread from North America to Europe. In Finland, the closest relative species of highbush blueberry cultivars (*V. corymbosum*) are bog whortleberry (*V. uliginosum*) and wild bilberry (*V. myrtillus*). All of these species can be

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classified as typical calcifuges, since they thrive in nutrient poor soils with pH about 5.5 or below (Korcak 1989).

Both cultivated and wild *Vaccinium*-species have special symbiotic mycorrhizal associations (ericoid mycorrhiza) in their root systems (Jacquemart 1996, Straker 1996). Mycorrhizas are considered mutualistic fungus-root associations (Bouchér et al. 1982), when their effect on both host plant and fungi fitness is positive and the net benefits of mycorrhizal symbiosis are greater than the net costs (Johnson et al. 1997). In ericoid mycorrhizas the mycorrhizal hyphae form coils in the epidermal root cells of host plant but do not penetrate the root endodermis or ensheath hair roots with mycorrhizal mantle (Smith and Read 1997, Deacon 1998). Benefits of ericoid mycorrhizas to host plant include increased nutrient and mineral uptake and tolerance to toxic substances like aluminium (Allen 1991, Read 1991).

Ericoid mycorrhizas are commonly found in roots of dwarf shrubs throughout temperate and boreal ecosystems (Bledsoe et al. 1990, Gardes and Dahlberg 1996), but there are still only few studies performed to investigate the intensity of ericoid mycorrhiza formation of plants growing in forests or plantings. The aim of the present study was to examine the mycorrhizal status of cultivated *Vaccinium corymbosum* as well as *V. uliginosum* and *V. myrtillus* growing in natural soils and to study especially the intensity of mycorrhizal development in *V. corymbosum* under agricultural field conditions. To study the exact localization and intracellular structure of the mycorrhizas in epidermal cells we used transmission electron microscopy (Duddridge and Read 1982, Smith and Read 1997). The mycorrhizal colonisation level of roots was estimated with two different methods, by biochemical ergosterol analysis and trypan blue staining combined with stereomicroscopy viewing. Ergosterol is a principal component of fungal membranes, and ergosterol assay is a general method used to determine the total fungal biomass in roots or soil (Wallander 1992). The root clearing and staining methods were originally developed for the assessment of vesicular-arbuscular mycorrhizas and parasitic fungi (Phillips and Hayman 1970), but with slight modifications these procedures are suitable for identification of ericoid mycorrhizas as well.

Material and methods

The two highbush blueberry (*V. corymbosum*) varieties investigated in this study were 'Northcountry' and 'Northblue', the most common cultivated highbush blueberries in Finland. Roots of wild bilberries (*V. myrtillus*) and bog whortleberries (*V. uliginosum*) grown under natural conditions were also studied. Root samples were collected twice, in September 1997 and early June 1998. 'Northcountry' and 'Northblue' root samples were collected from two different blueberry plantings near Muuruvesi (sites A and B, 63°00'N, 28°15'E) and from Kuopio University Garden (site C, 62°53'N, 27°37'E) in central Finland. Wild bilberries were sampled from naturally regenerated and spruce-dominated forests (podzol soil) in Kuopio and Muuruvesi, whereas root samples of bog whortleberries were collected only from a small *Sphagnum* bog in Kuopio. All sampled wild bilberries and bog whortleberries were growing in the vicinity of plantings.

In site A (farm Huumonen, Muuruvesi) the soil (fine sand) was treated with 80% sulphuric acid (sulphur dose 3.5 kg per are) one year before planting the blueberries in 1994, the obtained pH level being approximately 5.3 after this artificial acidification. In site B (farm Alatalo, Muuruvesi), pH level of the soil (fine sand + chips as a surface layer) was approximately 6.5 near the sampled plants, and the field established in 1992 was fertilised with Biolan Extra (NPK-ratio 4:1:2, dose 4 dl per plant) annually. In site C (Kuopio University Garden) cultivation had started already in 1987 and soil was adjusted for cultivars by applying peat and coarse sand to soil (loamy fine sand). Mean pH level of the soil was 5.5 and field was fertilised with Herkkuperunan

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lannos (NPK-ratio 8:10:12, dose 3–6 litres per are) annually from year 1995 onwards.

Highbush blueberry root samples were collected systematically, i.e. from every fifth plant in every second row, whereas wild bilberries and bog whortleberries were collected randomly from their growing sites. Roots were excavated from 0–20 centimetres depth with a small scoop, highbush blueberry roots being collected only from the part of the plant that faces south. After collecting, root samples were immediately rinsed under running tap water over a sieve. Then the hair root tips (length 2–3 mm) of washed roots were collected for ultrastructural study. Root samples for ergosterol analysis were frozen in liquid nitrogen prior storing them at –80°C, and the rest of the washed roots were stored at –20°C for the stereomicroscopy viewing.

The root tips collected for ultrastructural studies were placed immediately in 2.5% glutaraldehyde fixative in Eppendorf tubes. Prefixation was carried out in 2.5% glutaraldehyde made in 0.1 M phosphate buffer (pH 7.0) for 16 h at 4°C and postfixation in buffered 1% OsO_4 solution for 3 h at 4°C. The samples were dehydrated in graded ethanol series, infiltrated and finally embedded in Ladd's LX 112 resin. Then thin root sections were stained with uranyl acetate and lead citrate and mycorrhizal status of these sections was studied with a JEOL 1200 S electron microscope.

The gridline intersect method (Giovannetti and Mosse 1980) was used for visual estimation of the mycorrhizal infection in the hair roots. Approximately 50 milligrams (f. wt) of the finest hair roots (∅ < 100 µm) cleared in hot 10% KOH and acidified with 1% HCl were stained with 0.05% trypan blue in lactophenol (Phillips and Hayman 1970). After staining, the roots were dispersed as evenly as possible on a round Petri dish with help of glycerol. On the bottom of the Petri dish was a gridline (1 cm x 1 cm) and mycorrhizal colonisation status was studied from all the intersections of roots and gridlines.

Mycorrhizal occurrence in the roots was estimated also with ergosterol analysis (Salmanowicz and Nylund 1988, Nylund and Wallander 1992). Prior to the assay, the roots were freeze-dried and ground in liquid nitrogen. Ergosterol was extracted with ethanol containing pyrogallic acid from approximately 25–50 milligrams of root (d. wt). Saponification with 60% KOH released root ergosterol in free form, which was then extracted with pentane. Finally, pentane phase was left to evaporate to dryness and dissolved to methanol. HPLC-analysis was done using reverse-phase column (Hewlett-Packard, LiChrospher 100 RP-18) and 100% methanol as an eluent. Samples of 20 µl were injected and run at 1.6 ml per minute. Ergosterol peaks were detected with UV-detector at 280 nm, peaks appearing after 7–8 minutes. At the beginning and end of every sample sequence, internal ergosterol standards were run and regression formula for the standard curve was determined.

Mycorrhizal infection level data assessed both stereo- and electron microscopically was arcsin-transformed prior to the statistical tests (SPSS-PC-Windows programmes). One-way ANOVA combined with Tukey's test was used to analyse the differences between the mean infection levels and ergosterol concentrations of different groups. In addition, correlations between the root ergosterol concentration and mycorrhizal colonisation level data obtained from stereomicroscopical studies were tested with Pearson's correlation coefficient. In stereomicroscopical and ergosterol analyses the number of replicates was 10–25 (N = 107) and in ultrastructural analysis n = 5–10 (N = 45).

Results and discussion

In ultrastructural samples, mycorrhizal hyphae colonised epidermal root cells (Fig. 1) in all studied species. Although cultivars from site A (3.6%) and C (5.4%) had clearly the lowest ericoid mycorrhizal hyphae formation in epidermal root cells, the hyphae growth in cultivars from site B (27%) did not significantly differ from that of wild bilberries (47.9%) or bog whortleberries

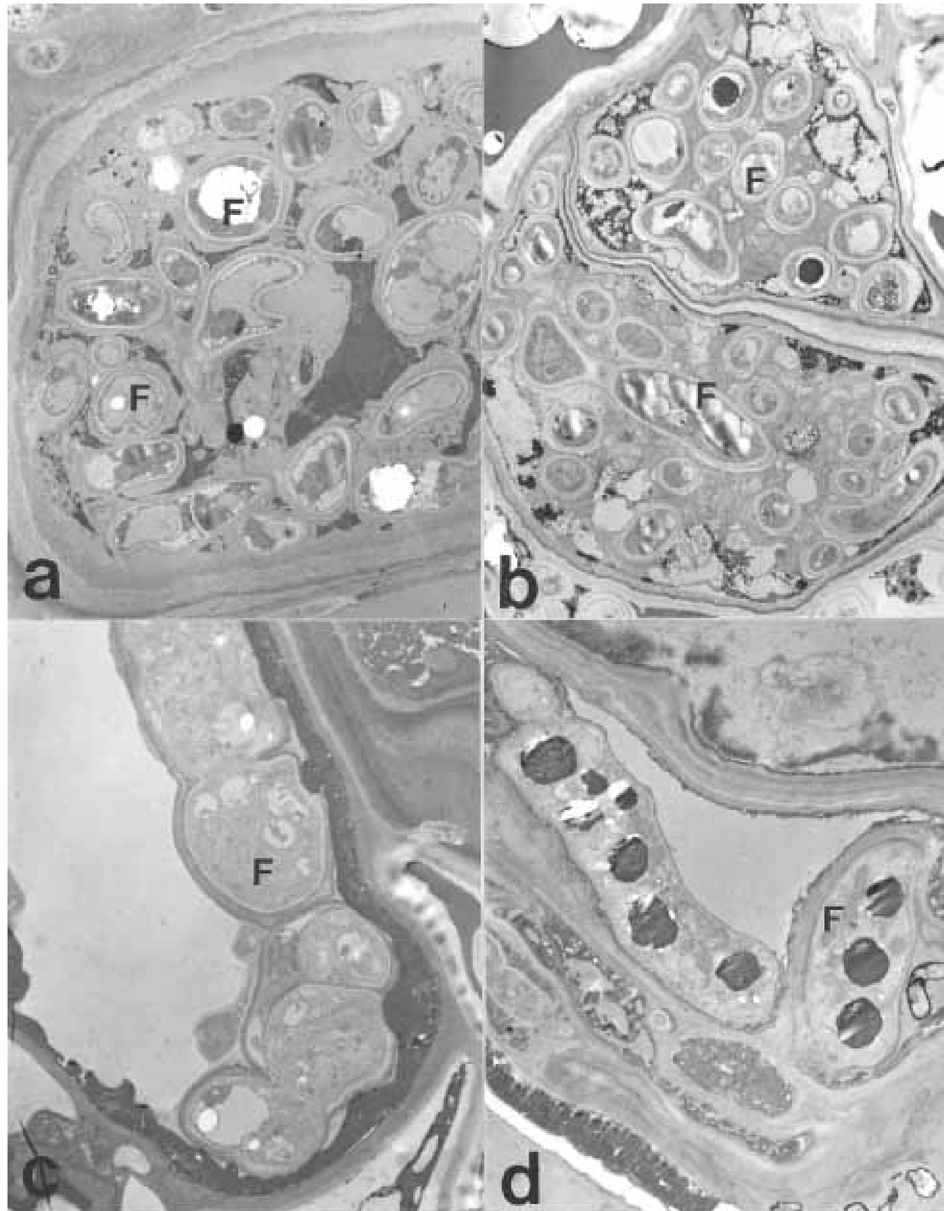

Fig. 1. Electron micrographs of ericoid mycorrhiza in highbush blueberry (a–b) and in wild bilberry (c–d) root epidermal cells. Well-developed mycorrhizal infection can be seen in the root epidermal cells, F = mycorrhizal cells. Magnification 7200x (a, c–d) or 5300x (b). Photo: Seija Anttonen and Toini Holopainen.

(20.8%). Previous transmission electron microscopic studies have revealed that in ericoid mycorrhizas each epidermal cell is an infection unit (Smith and Read 1997) and therefore even adjacent cells may have mycorrhizal complexes of different developmental stage. This pattern was

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observed also in the present material and the studied *Vaccinium* mycorrhizas represented the typical ericoid mycorrhiza structure (Smith and Read 1997).

Stereomicroscopical analysis showed that the mycorrhizal colonisation rate of roots was quite high in all studied groups in general, mean colonisation values ranging from 40% to 50.8% (Fig. 2a). This result is in accordance with the findings made by Johansson (2000), who discovered that naturally occurring ericoid mycorrhizal heather (*Calluna vulgaris*) had relatively high mycorrhizal infection level (approximately 40% of roots infected) during growing season. Total mycorrhizal infection percentage was lowest in bog whortleberries and cultivars from site C, whereas the highest total infection percentage was observed in wild bilberries (Fig. 2a). One explanation for the observed discrepancy between the TEM and stereomicroscopical data is that in ultrastuctural analysis only a small area of a two dimensional root section can be studied by TEM. Thus, in these small root sections the presence of infected cells is sporadic and therefore the results of TEM analysis can be regarded as more qualitative than quantitative. It is also important to remember that the visual assessment of mycorrhizas by stereomicroscope is a subjective method, in which some over- or underestimation of mycorrhizal status might easily occur, especially if the studied root sample is strongly pigmented.

The mean ergosterol content of roots in different study groups ranged from 71.3 µg g^{-1} to 153.7 µg g^{-1} (Fig. 2b). Contrary to the mycorrhizal infection data (Fig. 2a), bog whortleberries had clearly the highest amount of ergosterol in their roots. In addition, the cultivars from sites A and B as well as wild *Vaccinium*-species had clearly more ergosterol in their roots than the highbush blueberry cultivars in site C. In general, ergosterol assay revealed that there is a high total amount of vital mycorrhizal biomass in the roots of all investigated species. The ergosterol values observed in this study were approximately of the same magnitude or sometimes even greater than those found from ectomycorrhizal tree roots

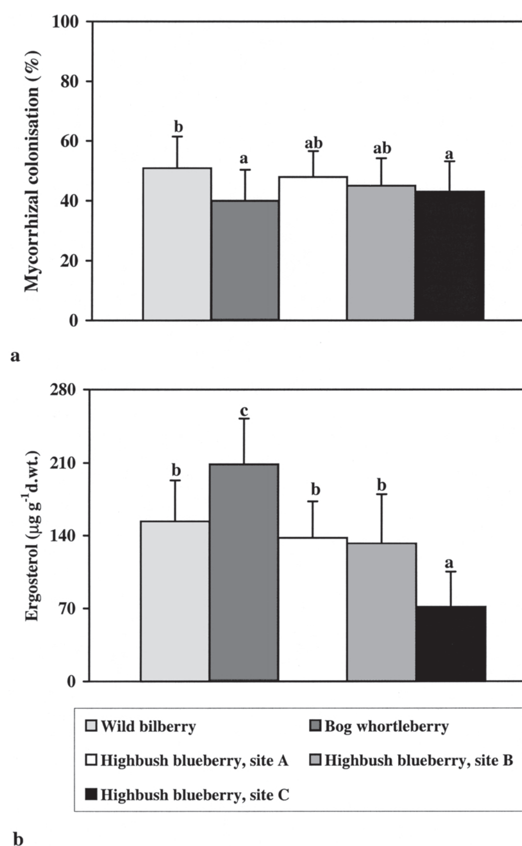

Fig. 2. a) Ericoid mycorrhizal colonisation levels (%, stereomicroscopic data) and b) ergosterol concentrations (µg g^{-1} of root d.wt.) in hair roots of wild bilberries, bog whortleberries and highbush blueberries in sites A, B and C. Site A = farm Huumonen Muuruvesi, site B = farm Alatalo Muuruvesi and site C = Kuopio University Garden, Kuopio. The values (mean ± SD) followed by the same letters are not significantly different from each other ($P < 0.05$).

(Ekblad et al. 1995, Manninen et al. 1998). The contradiction between ergosterol and mycorrhizal infection data might be partially due to the fact that ergosterol assay measures the total amount of living fungi (mycorrhizal plus non-mycorrhizal fungi), whereas in clearing and microscopic viewing method both dead and alive mycorrhizas are stained and counted. On the other hand, as Wallander et al. (1997) and Johnson and McGill (1990) point out, small seasonal variation in ergosterol amount and repeatability of

procedure makes it a better estimation method of vital mycorrhizal biomass than any other currently available analysis. Apparently because of the relatively large heterogeneity of data and possible above-mentioned inaccuracies caused by the methods used in this study, the ergosterol concentration and mycorrhizal colonisation of roots did not correlate significantly in any of the groups.

In conclusion, the highbush blueberry cultivars seem to obtain a substantial colonisation of ericoid mycorrhizal fungi from their growth medium. However, the infection level is somewhat lower compared to wild relative species and is probably dependent on the edaphic factors also. For instance, the soil medium in site C was more compact and loamy than in the other study areas and appeared to have a stunting effect on the growth of aboveground parts of plants as well. Previous studies have also shown that ergosterol concentrations can change according to the soil conditions (pH, nutrient and water status), fungus species, plant cultivars and age of fungus (Ekblad et al. 1995, 1998, Möttönen et al. 1999). Thus, it is possible that the ergosterol content of different plant roots varied merely due to the soil conditions and as mentioned earlier, edaphic conditions in site C seemed to control both mycorrhizal and plant growth. Moreover, the observed relatively high mycorrhizal formation in the studied cultivars does not necessarily mean greater benefits for the host plants. A further step would be to test experimentally whether the mycorrhizas infecting the roots are truly beneficial (mutualistic) to the highbush blueberries growing under agricultural field conditions.

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SELOSTUS

Viljellyn pensasmustikan ja luonnonvaraisten mustikan ja juolukan sienijuuret

Anne Kasurinen, Toini Holopainen ja Seija Anttonen

Kuopion yliopisto ja Metsäntutkimuslaitos

Viljellyn pensasmustikan (*Vaccinium corymbosum*) sekä luonnonvaraisten mustikan (*Vaccinium myrtillus*) ja juolukan (*Vaccinium uliginosum*) sienijuuria tutkittiin transmissioelektronimikroskoopilla (TEM), stereomikroskooppisesti trypaanisinisellä värjätyistä juurista ja biokemiallisella ergosterolianalyysillä. Kaikilta tutkituilta lajeilta löytyi erikoidimykoritsoille tyypillisiä rihmastokiehkuroita juuren pintakerroksen soluista TEM-analyysissä. Stereomikroskooppianalyysin perusteella luonnonvaraisilla mustikoilla oli korkeimmat mykoritsainfektiot juurissaan (hiusjuurista 51 % infektoitunut), kun taas suurimmat ergosterolipitoisuudet olivat juolukoilla (ergosterolia 209 µg/g juurta). Näiden tulosten perusteella voidaan sanoa, että mykoritsasymbioosit juurissa ovat yleisiä kaikilla tutkituilla *Vaccinium*-lajeilla, tosin viljellyillä pensasmustikoilla mykoritsainfektioiden määrä on luonnonvaraisia sukulaisiaan jonkin verran alempi.