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RESEARCH PAPER

Remarks on the spatial distribution of mycorrhiza in the roots of *Epipactis papillosa* (Orchidaceae)

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ABSTRACT

The article provides a description of the mycorrhiza in the roots of a generative specimen of *Epipactis papillosa* Franch. et Sav. (Orchidaceae). The percentage of bark cells inhabited by fungi and the intensity of mycorrhizal infection were calculated in all roots formed in 2010, 2011 and 2012 along all their length. Fungi were distributed in the roots irregularly, being concentrated in one or more zones. The possible connection between mycorrhizal development and weather conditions in corresponding years are discussed. Whereas according to the literature data *E. papillosa* is considered to be weakly mycotrophic species, with an average intensity of mycorrhizal infection of only 15 %, we found much higher average intensity in the studied specimen, up to 54 %. The possibility of secondary colonization of older roots by fungi in studied specimen was not confirmed.

Keywords: *Epipactis papillosa*, orchid mycorrhiza, orchid roots, mycoinfection, fungal symbionts, symbiosis

РЕЗЮМЕ

Кузьминова Л.С., Сальникова М.М., Виноградова Т.Н. К вопросу о распределении микоризы в корнях *Epipactis papillosa* (Orchidaceae). В статье приводится описание микоризообразования в корнях генеративного растения *Epipactis papillosa* Franch. et Sav. (Orchidaceae). Процент заселенных грибами клеток коры и интенсивность микоризной инфекции были подсчитаны для всех корней, образовавшихся в течение трех лет (2010–2012), по всей их длине. Грибная инфекция была распределена в корнях нерегулярно, концентрируясь в одной или нескольких зонах корня. Обсуждается возможная связь полученных данных с погодными условиями. Хотя, согласно литературным данным, этот вид относится к слабомикотрофным, со средним значением интенсивности микоризной инфекции 15 %, у изученного нами экземпляра этот показатель оказался гораздо выше и достигал 54 %. Возможность многократной колонизации корней микоризообразующими грибами у данного экземпляра представляется маловероятной.

Ключевые слова: *Epipactis papillosa*, микориза орхидных, корни орхидных, микоинфекция, симбиотические грибы, симбиоз

The orchid family (Orchidaceae) is the largest family of monocot plants, including up to 800 genera and 30,000 species (Tatarenko 1996, Givnish et al. 2015). 136 species from 43 genera grow in Russia (Vakhrameeva et al. 2014). Research on population ecology of orchids from different regions of Russia has continued over the past thirty years (Tatarenko 1996, Vakhrameeva et al. 2014).

The subject of the present study is a rare orchid *Epipactis papillosa* Franch. et Sav. Within Russia, its range includes Primorye, Amur and Sakhalin Regions, and Kamchatka Territory. Outside of Russia, it is found in Japan, China and in Korean Peninsula. *Epipactis papillosa* is a herbaceous perennial, usually 20–40 cm tall, sometimes as short as 10 cm (Tatarenko 1996) with ovate-lanceolate leaves covered with papillary hairs more distinctly than in closely related *E. helleborine* (L.) Crantz. Both taxa are considered closely related, and some authors even treat them as conspecific (Fateryga & Fateryga 2018). On the territory of Kamchatka, the species is preserved in the Nalychevo and Bystrinsky Nature Parks. It is also included in the official registry of protected species of Kamchatka and in the Red Data Book of Kamchatkskiy Krai (Chernyagina 2018), as well as in Appendix II of CITES.

The study of rare species is necessary both to develop measures for their protection and to assess the state of their populations. *Epipactis papillosa* is a poorly circumscribed species as concerns its population ecology, symbiotic interactions with other organisms, etc.

An integral part of an orchid life cycle are its symbiotic relationships with fungi, mycorrhiza: for some, only in the period after seed germination, and for most, throughout their life cycle (Phillips et al. 2011, Pecoraro et al. 2017). The intensity of mycorrhizal infection in the roots depends on various factors: availability of light, the chemical composition of the soil, the stage of plant development (Sizova & Vakhrameeva 1984, Tatarenko 1996). Thus, the percentage of cells populated by the fungus may be higher in plants that grow in a more shaded environment, as well as on soils with a higher content of organic matter. In younger (juvenile) plants, the mycoinfection will be higher than in generative ones (Bakulin et al. 2009). According to Betekhtina et al. (2013), the absolute abundance of fungi per unit length of the root does not demonstrate ontogenetic dynamics and rather depends on the exact place along the root length which was sampled.

The mycosymbionts of *Epipactis* species from *E. helleborine* aggregate belong to Ascomycete genera from the order

Pezizales, such as *Wilcoxina* Chin S. Yang & Korf, Tuber P. Micheli ex F.H. Wigg. and Hydnotrya Berk. & Broome (Ogura-Tsujita & Yukawa 2008). However, the intensity of mycorrhizal infection of representatives of this genus is still insufficiently explored. Existing literature on *E. helleborine*, which is closely related to *E. papillosa*, states that the amount of mycotrophic supply of nutrients in adult plants varies greatly and depends on growth conditions. Additionally, generative plants can be more mycotrophic than plants of other age groups (Vakhrameeva et al. 1997). Mycorrhizal development in *E. papillosa* has been studied by Tatarenko (1996), within Primorye Region and the Kuril Islands. In this work Tatarenko has shown that the intensity of mycorrhizal infection for this species was 15 %, and only in old dying roots it reached 80 %, with all hyphae completely digested. In the current study, we focus on the study of the changes in the intensity of mycorrhizal infection in the roots of different age. Since it is not possible to conduct long-term observations without serious damage to the studied organism, we simultaneously studied the roots of different age within one plant. In addition, we attempt to elucidate whether this species has secondary fungal colonization in 2–3-year old roots, or not.

MATERIAL AND METHODS

The studied *E. papillosa* specimen was collected in city roadside lawn in the Petropavlovsk-Kamchatsky, Bering Street, in September 2012 by Olga Chegeneva. The populations of *E. papillosa* from the City of Petropavlovsk-Kamchatsky are excluded from the Red Data Book of Kamchatka Territory (Chernyagina 2018), thus the collected specimen was not legally protected. The whole plant was fixed with 70 % alcohol. Subsequently all roots of this plant were sliced into a continuous series of temporal preparations. Slices were made manually using a straight razor at a distance approximately one millimeter from one another, and placed in glycerin, then placed on a glass slides in order, numbered continuously from the basal part of the root to the apical part. Since no part of root was wasted, the method enabled us to study mycoinfection along the whole root length.

The roots of different age were distinguished according to the markings left by the above-ground shoots of previous years. We distinguished roots which were formed in the years 2010, 2011 and 2012.

The rhizome section that grew during 2012 had six adventitious roots, the next section (2011) had four roots, and the last one (2010) had six roots. The length of the roots was 7 – 15 cm. Some of the roots (no. 2, 8, 13 and 15) were damaged and therefore fewer slices were made from them. In total, we studied 818 slices of which 293 were from roots formed in 2012, 205 from roots formed in 2011, and 320 from roots formed in 2010.

Slices were photographed using a DCM-310 digital camera and studied using a VM 301 microscope. The root diameter was measured with a ruler, and the cell sizes were measured with an object micrometer. The percentage of cortical cells inhabited by the fungus was counted in each slice.

Additionally, the intensity of mycorrhizal infection was calculated according to the standard method (Selivanov 1981), using the concept of "mycorrhizal score" characterizing the intensity of mycorrhizal infection following the formula

$$C = \frac{\sum_{i=1}^{i=5} n_i \times i}{N \times K}$$

where n_1 is the number of slices with a score of 1, n_2 – the number of slices with a score of 2, etc. Finally, N is the total number of slices examined, and K is the total number of scores (5). This measure reflects the relative abundance of the fungus in the root of the plant. A score of 1 means that the fungi are contained in very few cells of the cut bark; a score of 2 – fungi are contained in about a third of the cells of the cortex of the cut; 3 – in about a half of the cells; 4 – in more than two thirds of cells; 5 – in almost every cell of the cortex of the slice. This measuring method is less accurate than the calculation of the percentage of cells infected with the fungus, but it is widely used and this makes it possible to compare our results with the results of other authors.

RESULTS

Root anatomy

In the studied specimen, the root diameter varied from 1 to 4 mm, i.e., it was much larger than in previously studied juvenile plants of this species which were only 1.2–1.3 mm (Bakulin et al. 2009, Vinogradova & Kulikova 2012). The outer layer of root cells, a single-layer rhizodermis, consisted of cells 0.02 × 0.04–0.05 mm. Further in there were up to ten layers of rounded cells of the cortex. The smallest cells of the outer layers were approximately 0.05–0.07 mm in diameter. Larger cells of the inner layers of the cortex reached 0.10–0.15 mm in diameter. Finally, the layer of cells adjacent to the endodermis was 0.04 mm in diameter. This was very similar to the results obtained by Bakulin et al. (2009). Roots with larger diameter differed not so much in the number of cell layers, as in the size of these cells, the largest reaching 0.20 mm. Two to six xylem elements were visible in each root. Calcium oxalate raphides were found in some cells of the cortex. Finally, as has been shown for other orchids (Peterson & Currah 1990), fungi do not penetrate the meristem and the central cylinder.

In general, the anatomy of the roots of this species differed little from the anatomy of the roots of other species of the tribe Neottioideae, such as *Neottia cordata* (L.) Rich. (Vinogradova 1996).

Features of mycorrhiza development

Six roots formed during the last growing season, i.e. in 2012 (Fig. 1, A–F), were rather sparsely colonized with fungi, 0–14 %, 6.6 % on average, with C=5. In addition, only one zone of colonization (rarely two) was observed in this section of the roots. The colonization zone was located in the middle of the root, or closer to its apical part, where the proportion of bark cells populated by the fungus reached 80 %. The cells of the cortex contained numerous leucoplasts, which occupied almost the whole cells.

In the four roots formed during the preceding year, i.e. in 2011 (Fig. 1, G–J), fungi were present in the form of semi-

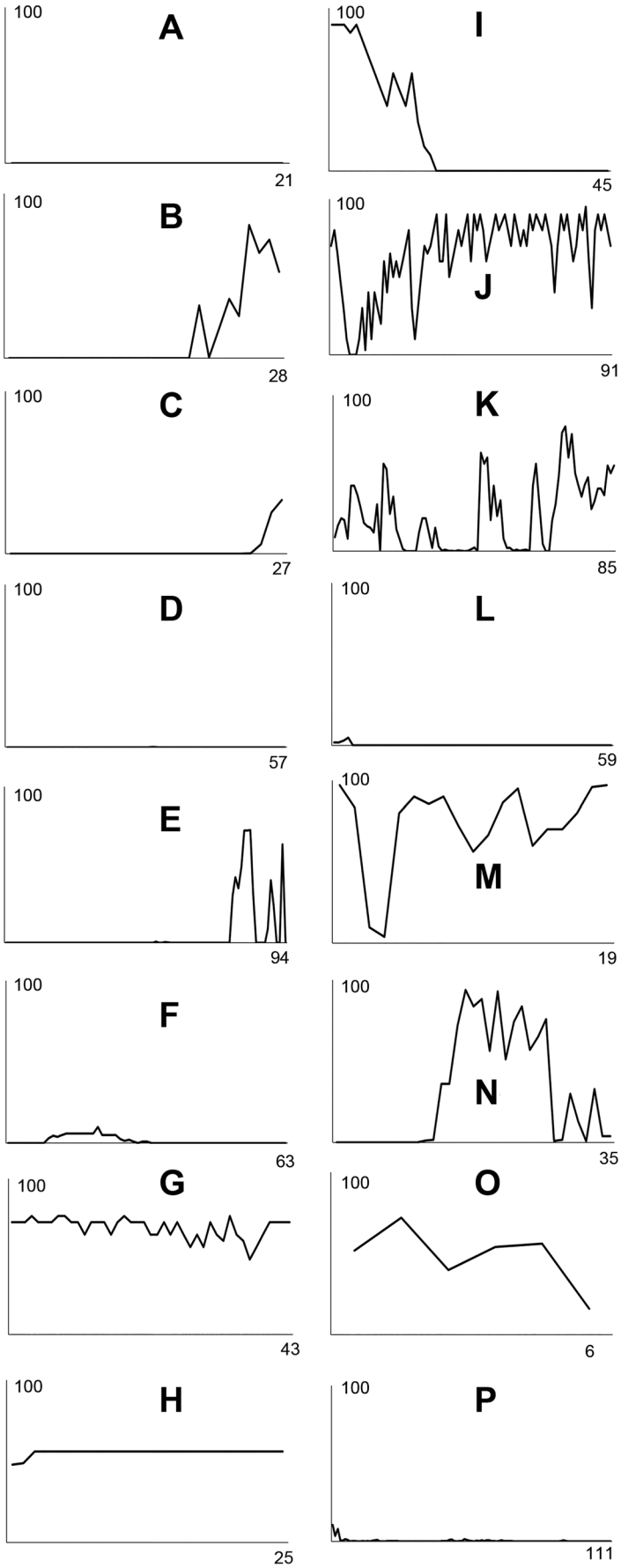

Figure 1 The proportion of cortical cells populated by fungi in the roots of the studied specimen of *Epipactis papillosa*. Vertical axis, proportion of cortical cells populated by fungi, %; horizontal axis, slices in the sequence from the basal to the apical part of the root. A–F, roots formed in 2012; G–J, roots formed in 2011; K–P, roots formed in 2010

digested yellow clumps, called pelotons, separated from the cell walls and occupying one half to one third of the cells. The proportion of cortical cells containing fungi was significantly higher, 20–70 %, 54.3 % on average, C=38. Fungi were distributed unevenly along the root, but almost throughout its length. Occasionally, we observed a narrow zone not inhabited by fungi.

The six oldest roots, i.e. those formed during the year 2010 (Fig. 1, K–P), were on average less colonized by fungi than the roots formed during 2011, 0–73 % of cortical cells, 21.5 % on average, C=32. Our examination of roots formed in the years 2010 and 2011 did not show a difference in the color and shape of pelotons, which would have indicated a varying degree of digestion

DISCUSSION

Both young and old roots turned to be populated with mycosymbionts to an unequal degree, from almost sterile ones (Fig. 1D) to rather strongly populated with fungi, with up to 68 % of cells infected (Fig. 1G). In the same time, most of the roots were colonized by fungi irregularly, with one or more zones with numerous pelotons, separated by zones without or with minimal fungal infection (Fig. 1B, E, K).

In previously studied juvenile specimens of this species (Bakulin et al. 2009, Vinogradova & Kulikova 2012), the intensity of mycorrhizal infection was found much higher, 75–95 %. This is not surprising, given that the intensity of mycorrhizal infection in orchids varies depending on life stage, decreasing from juvenile to generative specimens (Sizova & Vakhrameeva 1984). On the other hand, some of the earlier data showed an average of only C=15 % (Tatarenko 1996), which is much less than in the specimen studied here.

Since repeated colonization of roots by mycorrhizal fungi in juvenile specimens was observed earlier (Bakulin et al. 2009, Vinogradova & Kulikova 2012), we were interested to study whether this phenomenon was also observed in the studied specimen, which is at the generative life stage. Repeated colonization by fungi may be confirmed if there are the pelotons at different stages of digestion in the cells of the root cortex, and if the intensity of mycorrhizal infection is higher in older roots. However, the picture we see was different. Although the roots formed in the year 2012 were more weakly infected by fungi than the older ones, roots formed in 2010 and 2011 didn't differ substantially by the level of fungal infection. The intensity of mycorrhizal infection in the roots formed in 2012 was the lowest, but in the roots formed in 2010 it was somewhat lower than in 2011 (32 and 38%, respectively). This contrast is even more noticeable when comparing the proportion of populated cells – on average 21.54 % for the roots formed in 2010

Table 1. Average monthly temperature and precipitation (Climate… 2015).

Year	June		July		August	
	Average monthly temperature, ˚C	Precipitation, mm	Average monthly temperature, ˚C	Precipitation, mm	Average monthly temperature, ˚C	Precipitation, mm
norm	8.2	53	11.5	62	12.5	91
2010	10.2	36	13.0	72	14.3	52
2011	8.9	87	13.6	47	15.5	19
2012	11.3	21	13.8	68	15.2	12

and 54.3 % for the roots formed in 2011. All pelotons in older roots were morphologically similar, suggesting that they represented similar stages of digestion in the absence of secondary fungal colonization of older roots.

Thus, we got no evidence of possible recolonization of root cortex cells by fungi in the current study. Recolonization is still possible, if it occurs not occur every year, or in younger specimens. Nevertheless, the picture observed in the intensity of mycorrhizal infection between roots of different years is noteworthy.

For *Epipactis helleborine*, it has been noted that the mycotrophy or autotrophy of adult plants varies greatly and depends on growing conditions, to the point that generative plants can be more mycotrophic than plants of other age groups (Vakhrameeva et al. 1997). The growth conditions, such as variation in soil or light, did not change for our specimen from year to year, according to the information from the collector. In addition, despite being located street-side, it has not been artificially watered. In an attempt to account for variations in mycorrhizal infection over the three years, we compared the obtained results with temperature and precipitation data in Petropavlovsk-Kamchatsky for the summer months of 2010–2012 (Climate… 2015).

The summer of 2012 was the driest of the three, with only 101 mm of precipitation during the three summer months against about 216 mm of the norm (Table 1). It is possible that the low degree of fungal colonization of the roots which formed in that year was a result of this drought.

The number of days during which precipitation was observed in the summer of 2010 and 2011 were practically the same (38 and 42 out of 92, respectively). The total amount of precipitation was also not very different (160 mm in 2010 and 153 mm in 2011, Table 1), but the precipitation was distributed differently: in 2010, in June, precipitation was less than the average for this month, whereas in 2011, on the contrary, it was much more. This is consistent with the results obtained: the roots with maximum mycoinfection date back to the years with precipitation having its maximum at the beginning of the vegetation season. Therefore, it is possible that the most critical for the process of colonization of roots by fungi is the amount of precipitation at the beginning of the growing season.

However, as far as this study was done after a single plant, the observed parallelism between precipitation and mycoinfection needs further confirmation. More detailed additional studies using a statistically significant amount of material are needed.

We consider that the generally accepted method for calculating the intensity of mycorrhizal infection is less accurate than direct calculation of the percentage of cortical cells with fungi. In our case, the average percentage of cells inhabited by fungi in the roots formed in the years 2011 and 2010 differed more than twice, while the method proposed by Selivanov (1981) does not return such a large difference. The latter technique is convenient for quick processing of the large amount of material, while the calculation of the percentage of cells populated by fungi in each slice returns more precise and accurate data, but it is time and resource consuming and therefore cannot be universally recommended.

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