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Review

Successes and challenges in the sustainable cultivation of edible mycorrhizal fungi – furthering the dream

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ABSTRACT

The cultivation of edible mycorrhizal fungi (EMF) has made great progress since the first cultivation of *Tuber melanosporum* in 1977 but remains in its infancy. Five cultivation steps are required: (1) mycorrhizal synthesis, (2) mycorrhiza development and acclimation, (3) out-planting of mycorrhizal seedlings, (4) onset of fructification, and (5) performing tree orchards. We provide examples of successes and challenges associated with each step, including fruiting of the prestigious chanterelles in Japan recently. We highlight the challenges in establishing performing tree orchards. We report on the monitoring of two orchards established between *Lactarius deliciosus* (saffron milk cap) and pines in New Zealand. Saffron milk caps yields reached 0.4 and 1100 kg/ha under *Pinus radiata* and *P. sylvestris* 6 and 9 y after planting, respectively. Canopy closure began under *P. radiata* 7 y after planting, followed by a drastic reduction of yields, while *P. sylvestris* yields still hovered at 690 to 780 kg/ha after 11 y, without canopy closure. The establishment of full-scale field trials to predict yields is crucial to making the cultivation of EMF a reality in tomorrow's cropping landscape. Sustainable EMF cultivation utilizing trees in non-forested land could contribute to carbon storage, while providing revenue and other ecosystem services.

Keywords: ecosystem services, forest mushroom, *Lactarius deliciosus*, orchard, truffle

1. Introduction

Edible mycorrhizal fungi (EMF) include truffles (Périgord black truffle, Italian white truffle etc.) and other highly prized forest mushrooms (porcini, chanterelles, matsutake etc.). Most edible EMF truffle species belong to Ascomycota, while EMF mushrooms belong to Basidiomycota. They are abundant in boreal and temperate forests, where they live in ectomycorrhizal symbiosis with trees, colonizing their roots and transforming them into well-defined ectomycorrhizae, mixed organs where fungal and plant tissues are merged. EMF are, however, also present in dry and desert areas, e.g. the Mediterranean Basin, where the so-called 'desert truffles' engage in a polyvalent mycorrhizal relationship with Cistaceae Juss. of the genus *Helianthemum* Mill. (Morte, Gutiérrez, & Navarro Ródenas, 2020). Mycorrhizae enable both partners to live together and exchange mutual services (Smith & Read, 2008). The fungus supplies the tree with water and nutrients that its extensive mycelium, made of microscopic hyphae, pumps or mobilizes from the soil. In exchange, the tree, or shrub, reallocates photosynthates to its fungal symbiont, which in most cases, are otherwise inaccessible to EMF, given their inability to decompose carbohydrate polymers such as starch, cellulose or lignin. From a practical point of

view, this symbiotic relationship leads to (1) fast-growing and resilient mycorrhizal host trees (Selosse, Bouchard, Martin, & Le Tacon, 2000; Guerin-Laguette, Conventi, Ruiz, Plassard, & Mousain, 2003; Guerin-Laguette et al., 2014; Garbaye, 2013; Arteaga-León et al., 2018; Wang, Guerin-Laguette, Butler, Huang, & Yu, 2019a) and (2) an increased fungal biomass, both in the mycorrhizal roots and in soil mycelium (Read, 1991; Brundrett, Bougher, Dell, Grove, & Malajczuk, 1996). When conditions are appropriate, usually several times a year under natural conditions, fungal biomass in the soil supports the formation of mushroom or truffle fruiting bodies. These edible ascomata or basidiomata can be a source of food of high economic value (Watling, 1997).

Worldwide, EMF represent a multi-million dollar industry (Alexander, Pilz, Weber, Brown, & Rockwell, 2002; Hall, Wang, & Amicucci, 2003; Bonet, Gonzáles-Olabarria, & Martínez de Aragón, 2014). To date, the vast majority of commercial EMF are sourced from natural, wild ecosystems (Wang & Hall, 2004; Arora, 2008a; Hernandez Santiago et al., 2016; de Frutos, Rodríguez-Prado, Latorre, & Martínez-Peña, 2019). There are two main reasons: EMF are an important source of food and income for local human populations (Boa, 2004; De Roman & Boa, 2006; Arora, 2008b; Cai, Pettenella, & Vidale, 2011; Degreef et al., 2016; Liu et al., 2018), and most EMF species are not yet cultivable due to their complex symbiotic life cycle. High-value truffles such as *Tuber melanosporum* Vittad., *T. aestivum* (Wulfen) Spreng. & *T. borchii* Vittad. are an exception as they are now cultivated around the world (Hall, Brown,

& Zambonelli, 2007; Reyna & Garcia-Barreda, 2014). In the case of *T. melanosporum*, up to 90% of truffles produced in Europe come from man-made 'truffières' , French name for truffle orchards (Reyna & Garcia-Barreda, 2014; P. Sourzat, personal communication, Jun 4, 2020). In the southern hemisphere where *Tuber* P. Micheli ex F. H. Wigg. species were not originally present (Bonito et al., 2013), the totality of truffles in Australia, New Zealand, Chile and South Africa come from truffle orchards. However, truffle cultivation is very recent in human cropping history and additional research is needed to increase yields and profitability (Linde & Selmes, 2012; Reyna & Garcia-Barreda, 2014).

Truffles are considered the first EMF to ever be cultivated, accidentally at first, when farmers in the south of France were sowing acorns of oaks in soils that were naturally rich with truffle spores. Spores of truffles readily colonized young oak seedlings, and the young oak saplings starting to produce truffles a few years later (Olivier, Savignac, & Sourzat, 2018). In the mid twentieth century, following the steps of mycorrhiza synthesis pioneers (Norkrans, 1949; Hacskaylo, 1953; Trappe, 1967), European scientists began to master the controlled mycorrhization of tree seedlings with truffle spores (Palenzona, 1969; Grente, Chevalier, & Pollascek, 1972; Chevalier, Grente, & Pollascek, 1973). The concept of the EMF mycorrhizal seedling was born. In 1977, *T. melanosporum* truffles were harvested from 4.5-y-old out-planted *Corylus avellana* L. seedlings (Chevalier & Grente, 1978). This was the first successful controlled cultivation (i.e. man-induced fruiting-body production) of an EMF species based on science achievements, i.e. mycorrhizal synthesis. In comparison with some agricultural (wheat, rice) or horticultural (fruit) crops that have been cultivated over centuries or thousands of years (Balfourier et al., 2019), the cultivation of EMF is extremely recent in human history, exactly 43-y-old. Therefore, further basic and applied research is expected to improve success in the cultivation and production of EMF species.

There are many beneficial attributes of EMF cultivation. EMF are not only a delicious food, they also constitute an important source of minerals and amino-acids (Wang et al., 2014; Muszyńska, Kała, Firlej, & Sułkowska-Ziaja, 2016), a crucial property at a time our societies endeavour to increase the source of non-meat proteins (Gurbuz, 2019). Some species contain bioactive compounds of medical or nutraceutical importance (Pérez-Moreno & Martínez-Reyes, 2014). Besides producing a healthy food, EMF cultivation can help to protect wild fungal populations from overharvesting and potential extinction. Further, EMF tree orchards could be an attractive alternative, or addition, to animal product farming (e.g. dairy) or a complement to agroforestry in arable lands. Indeed, planting EMF trees in such lands could contribute towards global effort to mitigate carbon emissions since carbon-fixing perennial ectomycorrhizal trees are required to produce mushrooms and truffles. In addition, the belowground mycorrhizosphere is an important carbon sink (Churchland & Grayston, 2014) and ecosystems dominated by ectomycorrhizal fungi further increase soil carbon storage compared to arbuscular mycorrhizal fungi ecosystems (Averill, Turner, & Finzi, 2014). It has also been shown that the benefits provided by agroforestry systems are multiple, especially on grazed dairy systems (England, O'Grady, Fleming, Marais, & Mendham, 2020). Agroforestry is a promising approach to improve the resiliency of agricultural systems, protect the environment (Smith, Pearce, & Wolfe, 2012) and slow the effects of climate change by contributing to capture carbon (Brown, Miller, Ordonez, & Baylis, 2018). We suggest that integrating EMF trees in farms or in agroforestry is an attractive option that could add economic benefit with the production of marketable edible fungi. Similarly, there is an in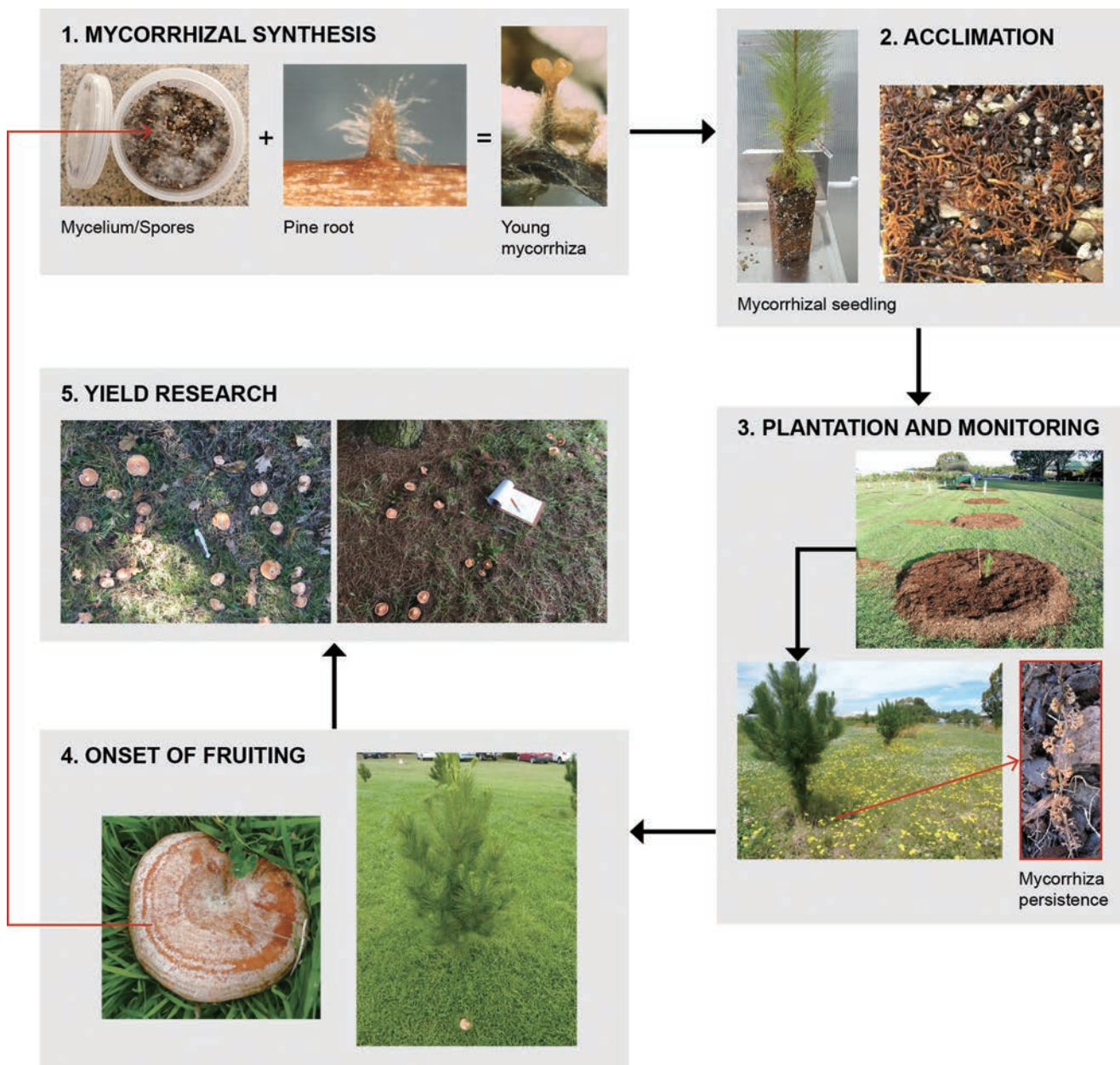creased recognition for the potential benefits of edible forests in marginal or transitional lands (Björklund, Eskvärd, & Schaffer, 2019) to which EMF trees would be a logical inclusion. Last but not least, the scientific breakthroughs required to master EMF cultivation accumulates basic knowledge concerning their life cycle, biology and ecology, a knowledge that can, in turn, be used to better protect wild EMF populations in their natural habitat, or provide a tool box to cultivate them in agroforestry systems. Beside cultivation in tree orchards, the research on production and management of wild populations of EMF (Savoie & Largeteau, 2011; Tomao, Bonet, Martínez de Aragón, & de-Miguel, 2017; Sun, Feng, Li, Shi, & Ding, 2019) could contribute to the sustainable use of forested land. To a certain extent, this area of research (cultivation and management of EMF) mirrors, on the land for forest edible fungi, the ongoing move in the sea towards sustainable management and farming techniques based on science and innovation (van Hoof et al., 2019).

Most EMF species are cultivated symbiotically, i.e. in association with a host plant. However, depending on the degree of mutualism, some species may be cultivated without a host plant. This is the case for *Lyophyllum shimeji* (Kawam.) Hongo, also known as Hon-shimeji that has a large commercial potential and is highly sought after in Japan. The saprotrophic cultivation of this EMF species without a host plant was pioneered by Ohta (1994) and the mushroom is nowadays commercially produced in Japan. A review of its cultivation history and methods, including symbiotic approaches, can be found in Yamada, Furukawa, & Yamanaka (2017). Although more EMF species may become one day cultivable without a host plant – through a better understanding of their physiological requirements – the present review will not cover this area of research.

The purpose of this review is to present the state of the art of the symbiotic cultivation of EMF 43 y following the initial success. This review will focus on the technical aspects: what is required to produce fruiting bodies of EMF, *de novo*, i.e. in a new piece of land starting from separated plant and fungal germplasms? What are the successes to date? What are the pitfalls? What are the next challenges and opportunities to make the cultivation of EMF more reliable, sustainable and profitable? The cultivation process is divided into the five following steps: (1) controlled mycorrhizal synthesis, (2) nursery acclimation of mycorrhizal seedlings, usually for 1 to 2 y, (3) orchard establishment: out-planting and monitoring of mycorrhizal seedlings in selected or prepared land, (4) onset of fruiting body formation and, (5) optimizing tree orchards and sustaining the yield of marketable fruiting bodies. For each step, examples of successes and challenges are provided, including original results. Finally, the results obtained by monitoring closely the yields of *Lactarius deliciosus* (saffron milk) caps in pine orchards in New Zealand highlight the need to document, optimize and sustain EMF yields. Research to understand and improve yields in tree orchards can be seen as the ultimate goal of any attempt to truly cultivate a given EMF species.

2. Controlled mycorrhizal synthesis

The cultivation cycle of EMF begins with controlled mycorrhizal synthesis, as illustrated in Figure 1. The principle is apparently simple: confront receptive non-mycorrhizal roots of a compatible host plant with a specific fungal inoculum in a suitable environment. Yet, realizing this objective can be complex and its outcome subject to many factors (Peterson & Chakravarty, 1991; Brundrett et al., 1996; Guerin-Laguette, Plassard, & Mousain, 2000a; Quoreshi, Kernaghan, & Hunt, 2008) some that may be unforeseen (See section 3) and some that may require third party organisms, such as

Fig. 1. – Cultivation cycle of an edible mycorrhizal fungus. The five steps involved are illustrated based on the example of *Lactarius deliciosus* (saffron milk cap) cultivated in association with *Pinus radiata* (Monterey pine).

mycorrhiza helper bacteria or plant growth-promoting rhizobacteria (Navarro-Rodénas, Berná, Lozano-Carillo, Andrino, & Morte, 2016). If the biotic and abiotic conditions of the environment are favourable to the particular requirements of the coupled symbionts, ectomycorrhizae will start developing in a matter of days (Fortin, Piché, & Lalonde, 1980; Guerin-Laguette, 1998; Marmeisse et al., 2004; Wang, Guerin-Laguette, & Yu, 2020) or weeks (Molina & Trappe, 1994; Hutchison, 1999; Guerin-Laguette et al., 2000b; Marozzi et al., 2017; Kinoshita, Obase, & Yamanaka, 2018) and the operation will be successful (Fig. 1). However, unfavourable conditions for either partner prevent a successful mycorrhization.

From a commercial application point of view, the preparation of receptive clean roots is best achieved by selecting seeds, cleaning and, in some cases disinfecting them (e.g. with H_2O_2 or NaClO) (Molinier et al., 2013; Wang et al., 2019a), and germinating them under clean but non-sterile conditions (Brundrett et al., 1996). This

entails using sterilized or pasteurized growing mixtures (by steaming or autoclaving) prepared in clean containers (free of mycorrhizal inoculum) in a greenhouse of the following hygiene standards: soil-free floor, grid tables, sufficient light, controlled temperature and water, and without other plants in the vicinity that could potentially be a source of contaminating non-target EMF. Although there is not enough scientific data to assess the effect of such practices, commercial glasshouses producing truffle seedlings are often thoroughly disinfected between different production batches, for example using didecyldimethylammonium chloride in Spain (M. Sánchez, personal communication, Jan 29, 2020). In cases where controlled *in vitro* mycorrhizal synthesis is required, root systems must be fully sterilized and raised in axenic conditions (Hacskaylo, 1953; Guerin-Laguette et al., 2000b).

Either vegetative mycelium (i.e. pure culture) or spores (i.e. usually spore suspensions or 'slurries') can be used as fungal inoc-

ulum to form ectomycorrhizae (Brundrett et al., 1996; Garbaye, 2013). Both approaches have their advantages and disadvantages (Brundrett et al., 1996; Fortin, Plenchette, & Piché, 2008; Garbaye, 2013). Pure cultures are often more expensive to produce on a large scale (Marx, Marrs, & Cordell, 2002) but they allow for greater strain precision and uniformity, and eliminate the risk of introducing undesired ectomycorrhizal species during the inoculation process (Garbaye, 2013; Iotti, Piattoni, Leonardi, Hall, & Zambonelli, 2016). A potential drawback for mycelial cultures of the ascomycete *Tuber* spp. is that they are haploid, thus contain only one mating type (Paolocci, Rubini, Riccioni, & Arcioni, 2006; Riccioni et al., 2008; Rubini et al., 2011; Kües & Martin, 2011; Taschen et al., 2016) while two mating types are required for truffle formation (Le Tacon et al., 2016). Basidiomycete EMF cultures can be obtained from fruiting bodies or mycorrhiza tissues and are heterokaryotic, therefore self-fertile since resulting from the merger of two compatible monokaryons (Clémençon, 2004; Garbaye, 2013). However, not all basidiomycete EMF can be readily cultured (Garbaye, 2013). The successful propagation of fruiting bodies of specific genera is notoriously either challenging, e.g. *Cantharellus* Juss. and *Hydnum* L. (Sugawara et al., 2019) *Russula* Pers. and *Lactifluus* (Pers.) Roussel (Wang & Guerin-Laguette, unpublished), or the mycelium obtained grows slowly, e.g. *Amanita* Pers. (Daza et al., 2006) or can be difficult to maintain, e.g. *Boletus* L. (Endo et al., 2014). Aside from high-valued truffle species, spore inocula are generally less expensive to produce (affordable, easy to prepare and store, no pure culture equipment required) and are easier to apply to a large number of seedlings (Marx et al., 2002; Fortin et al., 2008; Morte et al., 2017). For this reason, when attempting to initiate a mycorrhizal synthesis with an EMF species for the first time, spore inoculum is the cheapest option (Fortin et al., 2008). However, the fruiting-bodies from which spore inocula have been extracted can be contaminated by residual spores of other fungal species, or composed of a mix of species that are similar in appearance and difficult to distinguish (Hall et al., 2007; Murat, 2015; Iotti et al., 2016). For example, surface disinfection (Palenzona, 1969) and molecular testing of all ascomata used to prepare the inoculum are crucial in preventing co-inoculation of non-target truffle species during truffle seedling production (Murat, 2015). In the case of spore slurries, the ultimate screening against lookalike species would be next generation sequencing (NGS) to identify all fungi present in a sample. However, for more practical use, a panel of qPCR assays can be used to detect or rule out a range of undesired truffle species, with superior sensitivity to conventional PCR assays (J. Mackay, personal communication, May 22, 2020).

Depending on the target EMF species used, the type of inoculum can be a decisive factor for the outcome of the inoculation process. For example, for the pine-specific *Lactarius hatsudake* Nobuj. Tanaka & *Lactarius vividus* X. H. Wang, Nuytinck & Verbeken, vegetative mycelium readily produces mycorrhizae while spore inoculum derived from the same fruiting-bodies, i.e. those that were used to obtain the pure culture mycelium, fails to produce mycorrhizae (Wang et al., 2019b). This same failure of mycorrhization from spores has also been observed with *Lactarius deliciosus* (L.) Gray and young seedlings in the nursery (Guerin-Laguette & Wang, unpublished). The reason for this is unknown but spore dormancy could be involved (Wang et al., 2019b). In New Zealand, we speculate that spores of *L. deliciosus* successfully colonized established pine trees growing up to 1 km away from *L. deliciosus* tree orchards. This phenomenon was observed twice; once near Nelson (H. Krummenacher, personal communication, May 2014) and again at the Lincoln farm of The New Zealand Institute for Plant & Food Research Ltd (PFR) where a saffron milk cap fruiting-body

was found in May 2019 (Guerin-Laguette, unpublished) growing under *Pinus pinea* L. within a *T. borchii* truffière adjacent to a *L. deliciosus* orchard established in 2005. Spore dissemination (by wind, animals, water) from mushrooms produced by the orchard trees is the most likely explanation for the observed fruiting of *L. deliciosus* under distant *T. borchii* trees (about 300 m from the saffron milk cap trees in that particular example), since *L. deliciosus* is still largely absent from the wild in New Zealand. This species was first introduced into New Zealand in 1997 (Wang et al., 2002) and is still likely mostly restricted to *L. deliciosus* pine orchards (Wang, Cummings, & Guerin-Laguette, 2012). Surveys in the vicinity (within 1–2 km radius) of orchards would help understanding the presence of *L. deliciosus* outside orchards' boundaries. Based on my observations and those of Hannes Krummenacher, saffron milk mushroom production from initially non-inoculated, existing trees (outside orchards) may have started between 10 to 15 y after the establishment of nearby saffron milk cap orchards. It is likely that the spore-mediated spread and natural establishment of *L. deliciosus* outside the boundaries of *L. deliciosus* pine orchards has happened more than twice given that *L. deliciosus* orchards have been established across New Zealand since early 2000s (Wang et al., 2012).

2.1. Mycelial inoculum

Mycelial inoculum is usually obtained by propagating the flesh of EMF fruiting bodies (Brundrett et al., 1996; Daza et al., 2006) and more rarely from spores (Ohta, 1986; Murata et al., 2015). Recently, however, field-collected ectomycorrhizae (Ogawa et al., 2019a; Endo et al., 2020) and spores (Yamada et al., 2019; Sugawara et al., 2019) have been shown to provide valuable alternative sources of pure mycelial cultures. Nutrient-rich media, having high concentrations of exogenous glucose (1–20 g/L), are often required to sustain mycelial growth (Gibson & Deacon, 1990; Hutchison & Piché, 1994; Yamada, Ogura, & Ohmasa, 2001a; Daza et al., 2006). Although pure culture inoculum is grown under aseptic conditions, mycorrhizal synthesis is improved if root systems are inoculated in clean but non-sterile conditions, as described above for the germination of seedlings; the inoculation and growth of plants is achieved in the open (non-sterile) air under glasshouse conditions. Indeed, since the mycorrhizal synthesis aims to produce vigorous mycorrhizal seedlings that are fit for planting in the field, it is desirable to control the mycorrhizal synthesis under more 'natural' conditions, i.e. to bring about mycorrhiza synthesis in 'open', non-aseptic conditions. In such non-sterile conditions, both partners can develop vigorously *in vivo* from the inoculation stage and avoid the shock of a sudden transfer from aseptic to open conditions. So far, mycelial inoculum has only been successfully used to mycorrhize a small number of EMF basidiomycete species under such open-air (non-sterile) conditions: *Suillus luteus* (L.) Roussel, *S. granulatus* (L.) Roussel, *Tricholoma flavovirens* (Pers.) S. Lundell and *Boletus aereus* Bull. (Poitou, Mamoun, & Delmas, 1982), *L. deliciosus* (Poitou et al., 1982; Guerin-Laguette et al., 2000a; Wang et al., 2002), *Lactarius* sect. *Deliciosi* (Fr.:Fr.) Redeuilh, Verbeken & Walleyn (Wang et al., 2019b), and *Rhizopogon roseolus* (Corda) Th. Fr. (Visnovsky, Guerin-Laguette, Wang, & Pitman, 2010). For *L. deliciosus*, the efficacy of the mycelial inoculum is such that short roots newly generated on established pines can be successfully colonized by *L. deliciosus* in the field following the method of Guerin-Laguette, Matsushita, Lapeyrie, Shindo, & Suzuki (2005) (Wang, Cummings, & Guerin-Laguette, 2011). Numerous mycelial inoculum studies involving EMF have used *in vitro* conditions from the inoculation stage (Danell, 1994; Yamada et al., 2001a; Yamada,

Ogura, & Ohmasa, 2001b; review by Wang et al., 2012; Endo et al., 2014; Ogawa et al., 2019b; Horimai et al., 2020). To avoid the shock of a transfer from aseptic to open conditions and to produce mycorrhizal seedlings fit for out-planting, it appears desirable to achieve mycorrhizal synthesis and development from pure mycelial inoculum using clean, non-sterile conditions from the inoculation stage.

For several EMF species, successful mycorrhizal synthesis was so far only obtained, and probably only attempted, *in vitro*. This is the case of basidiomycete species: *Boletus edulis* Bull. (Agueda et al., 2008; Endo et al., 2014), *Tricholoma matsutake* (S. Ito & S. Imai) Singer (see review by Wang et al., 2012; Saito et al., 2018), *L. shimeji* (Visnovsky et al., 2014), *Amanita caesareoides* Lj. N. Vassiljeva and related species (Endo, Gisusi, Fukuda, & Yamada, 2013), *Astraeus hygrometricus* Pers. Morgan (Fangfuk et al., 2010) and *Cantharellus cibarius* Fr. (Danell, 1994; Ogawa et al., 2019b). However, in most cases, releasing *in vitro*-mycorrhized seedlings into non-sterile environments (e.g. glasshouse conditions) leads to the persistence of mycorrhizae over several mo (Endo et al., 2013, 2014; Yamada, personal communication, Dec 2017) and, more often, to the progressive collapse of the target species and replacement by other ectomycorrhizal fungi (Hall, Lyon, Wang, & Sinclair, 1998; Wang et al., 2012). The reasons for the instability of the associations obtained *in vitro* are unknown. Another drawback of mycorrhizal syntheses performed *in vitro* is that they may represent 'artificial' associations having little relevance to natural conditions (Yamanaka et al., 2014). However, new work suggests that *in vitro* mycorrhization methods can generate seedlings fit for out-planting. Recently, for example, mycorrhized oak and pine seedlings were synthesized *in vitro* from mycelial cultures of the truffle *T. borchii*. These seedlings were acclimatized in the glasshouse prior to out-planting in the field, where they successfully produced truffles eight years later (Iotti et al., 2016).
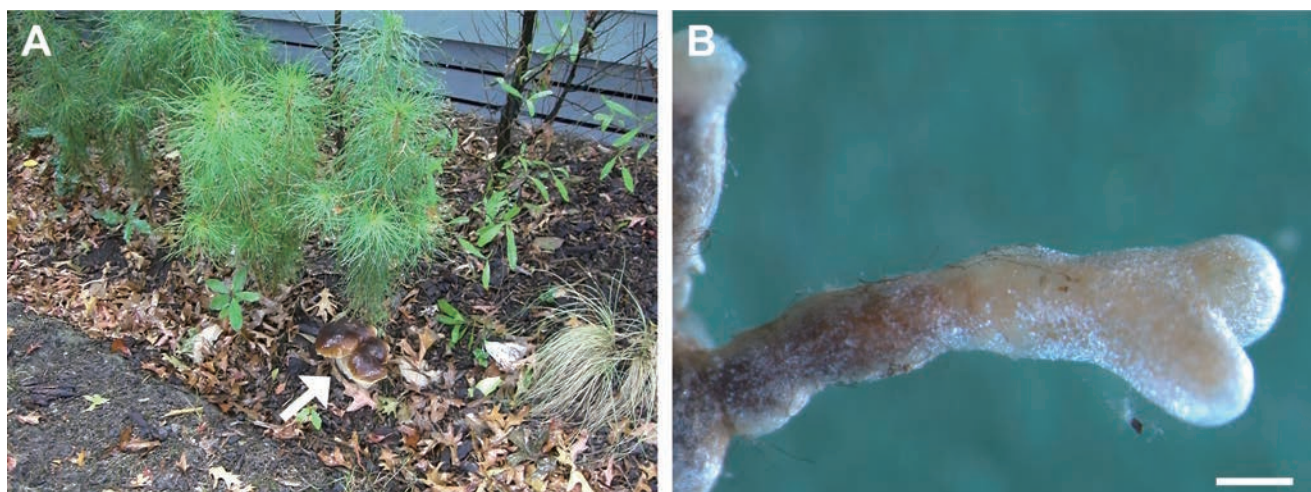
Even when the mycorrhizal association is proven to start and thrive under non-sterile conditions at the inoculation stage, e.g. *Lactarius* sect. *Deliciosi*, simple environmental issues can jeopardize the outcome of the inoculation process. For example, the mycelium inoculum of some *L. deliciosus* isolates can be very sensitive to the temperature within the nursery container; root temperatures of potted plants exceeding 26 °C may delay or inhibit the formation of ectomycorrhizae (Wang R et al., unpublished).

Mycelial inoculum can also be obtained from non-sterile sources, i.e. excised mycorrhizal roots of previously mycorrhized seedlings or of trees naturally associated with EMF in the field. This technique is referred to as the "mother plant technique" (Hall et al., 1998), a modification of the technique used by Joseph Talon for pioneering truffle cultivation in France in early 1800s (Olivier et al., 2018). In New Zealand, *Pinus radiata* D. Don (Monterey pine) seedlings were thus successfully mycorrhized by *B. edulis* by growing under porcini productive oak trees at the University of Canterbury, Christchurch for up to 5 y (Wang Y. et al., unpublished, Fig. 2A). Mycorrhization of the seedlings was confirmed by morphological (Fig. 2B) and DNA analysis, and by tracing mycorrhizal roots back to the stem of the pine seedlings (Guerin-Laguette, Cummings, & Wang, 2011). These seedlings were planted in 2007 in a lawn environment on the campus of PFR. In 2010, mycorrhizae of *B. edulis* were still detected on out-planted seedlings. However, no porcini mushrooms have been produced to date (Wang & Guerin-Laguette, unpublished).

2.2. Spore inoculum

Spore inoculum is usually applied to the root system under clean, non-sterile conditions. This type of inoculum is extensively used in the truffle industry worldwide (Murat, 2015; Morte et al., 2017). With the advent of molecular tools and improved taxonomy, it is now possible to ensure that undesired truffle species no longer colonize seedlings accidentally. Mycorrhizae obtained by this method with major commercial truffle species are shown in Figure 3, with illustrations of the fruiting-bodies produced. However, not all truffle species can be successfully inoculated using this method. The best example is *Tuber magnatum* Picco, the world's most expensive truffle. Only a mycelium-based inoculation technique has been successful in producing mycorrhizal seedlings (see section 7.2, Recalcitrant species). Scientists have also shown the potential of common mycorrhizal networks for producing truffle seedlings grown around spore-inoculated nurse plants (Pereira et al., 2013). Spore inoculum has also been used successfully for several edible basidiomycete mushroom species including *Rhizopogon rubescens* (Tul. & C. Tul.) Tul. & C. Tul. (Wang et al., 2002), *Hebeloma* (Fr.) P. Kumm. spp. (Martinez-Reyes et al., 2012; Carrasco-Hernandez et al., 2015), *Laccaria* Berk. & Broome spp. (Rentería-Chávez, Pérez-Moreno, Cetina-Alcalá, Ferrera-Cerrato, & Xoconostle-Cázares, 2017; Villegas-Oliveira et al., 2017) and *Helvella* cf. *lacunosa* Afzel. (Arteaga-León et al., 2018). Given the rich diversity of EMF

Fig. 2. – Mother plant technique used to inoculate *Pinus radiata* seedlings with naturally occurring *Boletus edulis* (porcini). A: *P. radiata* seedlings growing underneath porcini-productive oak trees at the University of Canterbury, Christchurch, New Zealand (2007). Note the fruiting bodies of porcini (arrow) growing next to the pine seedlings. B: Ectomycorrhiza of *B. edulis* formed on an unearthed *P. radiata* seedling. *Bar*: 190 μm.

worldwide, estimated to be over 500 species (Pérez-Moreno & Martínez-Reyes, 2014), the spore inoculation potential of valuable EMF species not studied yet for mycorrhizal synthesis and cultivation should be investigated.

Recently, spores and pure culture mycelium of *T. matsutake* were combined to inoculate *Pinus densiflora* Siebold & Zucc. seedlings *in vitro* in order to improve the mycorrhization and the development of a shiro-like structure (A. Yamada, personal communication, Jun 10, 2020).

3. Acclimation in the nursery

3.1. Issues affecting the development of mycorrhizae during acclimation in the nursery

Once ectomycorrhizae of a given EMF species are synthesized on a seedling, the next step is to ensure that they develop continuously throughout the root system of their host plant (Fig. 1). It is desirable to plant seedlings harbouring large quantities of mycorrhizae of the target species as one may assume that the higher the initial quantity of target mycorrhizae, the higher the chance of successful persistence and development of the mycorrhization by the desired species under field conditions, although this has not been demonstrated to date. Acclimation is relatively straightforward if basic environmental conditions are met such as temperature and moisture control, light intensity, cleanliness of premises and an appropriate potting mix. However, even under such controlled conditions, unanticipated phenomena may occur. For example, insect grazing (Wang et al., 2019b) or seedling growth issues, especially for pine seedlings inoculated with truffles under high pH (Guerin-Laguette, unpublished) may jeopardize the acclimation step. Recently, Leonardi et al. (2020) showed that high pH-induced chlorosis of *T. borchii*-inoculated *Quercus robur* L. seedlings could be reduced by the addition of biogenerated ferric hydroxides nanoparticles in the potting medium.

However, one of the best examples of an unforeseen issue during acclimation is probably the accidental cross-contamination between commercial truffle species in the nursery. Recent research carried out by PFR has shown, for the first time, that truffle seedlings inoculated separately with distinct truffle species could be sensitive to cross-contamination. Since 2015, Southern Woods Nursery (SWN, Templeton, New Zealand) has contracted PFR to carry out an independent quality control of their truffle mycorrhizal seedlings. Seedlings were inoculated separately with either *T. melanosporum*, *T. borchii* or *T. aestivum*. The inoculation material (blender, pipette tips) was carefully washed free of spores between different batches. Individually containerized seedlings were grouped into crates resting over grids and grown in the same greenhouse with no physical separation between various tree/truffle combinations. Seedlings inoculated with different truffle species (in separate containers) could thus be distant from each other by only a few cm (in two adjacent crates) or by a few metres. When testing the seedlings by microscopy to determine the outcome of the inoculation, we observed the repeated occurrence of *T. borchii*-like mycorrhizae on seedlings that were inoculated with either *T. melanosporum* or *T. aestivum* (Fig. 3A,D). DNA analysis using *T. borchii*-specific primers (Amicucci, Zambonelli, Giomaro, Potenza, & Stocchi, 1998) confirmed that these mycorrhizae were of *T. borchii*. Seven out of nine production batches of either *T. melanosporum* or *T. aestivum* had between 20 to 70% of their tested seedlings thus 'accidentally' colonized by *T. borchii* (Guerin, 2015). Such seedlings were therefore displaying two types of mycorrhizae: the target species that they were inoculated with and *T. borchii* mycor-

rhizae (Fig. 3D). This phenomenon has occurred every year in the nursery since 2015. However, since 2017, *T. borchii* seedlings are kept further away from the other two truffle species (by ≈ 5 m) and are also separated by a mesh wall reducing insect movement. Since 2017, the proportion of seedlings accidentally colonized by *T. borchii* at SWN reduced significantly: in the latest production season (2017/2018), none of the *T. aestivum*-inoculated seedlings showed *T. borchii* mycorrhizae while only four out of 13 batches of *T. melanosporum*- inoculated seedlings had 3 to 12% of their tested seedlings showing *T. borchii* mycorrhizae (Guerin, 2018). Given these observations and the common presence of live insects (springtails, spiders, worms) in the root ball of seedlings, it is possible that insect movement between seedlings is one likely source of cross-contamination by *T. borchii*. Indeed, insects could transfer to seedlings spores sticking to their bodies or transiting through their guts. Accidental incursion either of *T. melanosporum* or *T. aestivum* on other seedlings has not been observed and this may be because *T. borchii* are more aggressive and efficient in forming mycorrhizae via spore inocula than those of the other two truffle species. It is also possible that *T. borchii* may attract more insects or specific ones. However, another possible explanation would be that *T. borchii* produce conidia (asexual spores) that could be vectored by water or air, released in the vicinity of seedlings and contaminate seedlings on which they land. Urban, Neuner-Plattner, Krisai-Greilhuber, & Haselwandter (2004) demonstrated that *T. borchii* forms anamorphs (i.e. asexual state of fungi) producing mitotic spores that could represent an alternative dispersal mechanism independent from the well-documented endozoochory of truffles (Maser, Trappe & Nussbaum, 1978). So far, anamorphs have been found for two *Tuber* species only: *T. borchii* and *Tuber oligospermum* (Tul. & C. Tul.) Trappe (Urban et al., 2004). The lack, or rarity, of anamorphs in *T. melanosporum* and *T. aestivum* would explain why no cross-contamination with these species has been observed in our study.

Independently from these observations at the nursery stage, available information suggests that the same phenomenon has happened previously in New Zealand and overseas. In 2012, a New Zealand truffle grower reported that his Burgundy orchard was producing *T. borchii* in addition to *T. aestivum* (Gareth Renowden, personal communication, Apr 2012), a finding confirmed by PCR analysis of the ascoma (Guerin-Laguette, unpublished) and regular fruiting of both species in the orchard since. At first, a mix-up of seedlings at the nursery that supplied the truffle trees (a nursery different from SWN) was suspected as the most likely cause for dual fruiting. Then, in 2015, while testing mycorrhizae of a *T. melanosporum* orchard established in 2012 in Wanaka (Otago, New Zealand), mycorrhizae of *T. borchii* were detected and confirmed by PCR analysis (Guerin, 2016). Overseas, Berch & Bonito (2014) similarly found unexpected *T. borchii* mycorrhizae in British Columbia's *T. melanosporum* truffle orchards and hypothesized that seedlings had probably been mixed up at the nursery. The potential for cross-contamination of nursery seedlings resulting from the transport of *T. borchii* spores by insects and/or from the release of asexual mitospores of *T. borchii* should now be tested experimentally under controlled growing conditions. Our nursery observations suggest that trees inoculated with *T. borchii* should be grown separately from other truffle species seedlings, i.e. in a physically distinct and separated glasshouse space. No such precaution seems to be required for *T. melanosporum* and *T. aestivum* seedlings.

3.2. Mycorrhizal seedling certification

From a cultivation point of view, the 'end product' of the accli-

Fig. 3. – Ectomycorrhizae and fruiting-bodies of commercial truffle species in New Zealand. A–C: Ectomycorrhizae of *Tuber melanosporum* × *Quercus robur*, *T. aestivum* × *Q. ilex*, and *T. borchii* × *Pinus pinea* formed by spore inoculation at Southern Woods Nursery, Templeton, New Zealand. D: Accidental presence of *T. borchii* mycorrhizae (arrowhead) co-occurring with *T. aestivum* ectomycorrhizae (arrows) on a *T. aestivum*-inoculated *Q. robur* seedling. E, F: High-value truffles cultivated in truffières in Canterbury, New Zealand: *T. melanosporum* with truffle dog Cassie (Tewnion Truffière, Old West Coast Road, Christchurch) and *T. borchii* (harvested from the trial orchard at the Lincoln Farm of The New Zealand Institute for Plant & Food Research Ltd, Lincoln), respectively. *Bars*: A 550 µm; B 440 µm; C 350 µm; D 580 µm.

mation stage is a mycorrhizal seedling that is deemed suitable to establish an orchard that can produce fruiting bodies of the target EMF species. For large-scale nurseries, much work is still required to increase the mycorrhization of seedlings at decreased production costs. Scaling-up a production system while maintaining tree quality is still very much a challenge for most EMF species. The way forward is to develop certification schemes for guaranteeing, as much as possible, the quality of mycorrhizal seedlings produced in the greenhouse. Determining whether a given seedling is suitable for out-planting is still partially known and a complex task.

The following key criteria should be realistically checked by trained and experienced staff on seedlings (Andrés-Alpuente, Sánchez, Martín, Aguirre, & Barriuso, 2014): unequivocal confirmation of the presence of the target ectomycorrhizae, assessment of development of the target ectomycorrhizae (there are several methods, destructive or not), confirmation that ectomycorrhizae of non-target species are either undetected or in quantities lower than those of the target species. Further, seedlings should be healthy, free from obvious pathogens, and should have a vigorous and well-formed root system and stem. Certification schemes exist in several countries but more work is required to standardize or improve current certification methods (Andrés-Alpuente et al., 2014; Murat, 2015; Donnini, Benucci, Bencivenga, & Baciarelli-Falini, 2014). More field research is also required to determine the impact of the initial rates of mycorrhization by the target species, or of the presence of co-habiting non-target mycorrhizal species on the performance of the seedlings in the field in terms of production of the target EMF species (Guerin-Laguette et al., 2014).

4. Planting (EMF orchard establishment) and monitoring

Under an intensive cultivation model, the goal is to create an EMF tree orchard that will produce abundant quantities of the target mushroom or truffle species every year and over a long period of time. It is also possible to consider an extensive EMF cropping model, in which high numbers of mycorrhizal seedlings will be planted over a large area but with a minimal management (no tree protection, no irrigation). This approach would have a different focus, such as contributing to reforestation, increasing the volume of valuable edible fungi that could be harvested from the land by local communities, and could be seen as a silvicultural management technique to compensate for over harvesting of wild fungi and habitat disturbance (Arnolds, 1995; Mortimer et al., 2012). Such an approach would require the development of cost-efficient mycorrhization technologies for the large-scale production of seedlings. These technologies are not yet available for most high-value EMF.

In the intensive cultivation approach, many factors need to be considered when determining the suitability of a land to establish a commercial EMF orchard. The following fundamental requirements can be enumerated: a soil and climatic environment compatible with the tree and fungal species chosen; mycorrhizal seedlings of high quality; a soil devoid of – or with a low presence of – potentially competing ectomycorrhizal fungi; a management plan for the successful transplantation of seedlings (irrigation if required, physical protection against grazing animals, weed control until seedlings successfully outgrow the grass layer, mulching); a minimal distance between the orchard and neighbouring trees harbouring potentially competing ECM fungi; wind-breaks or shelter (e.g. arbuscular mycorrhizal fungi trees that won't be a source of potential contamination for the EMF orchard) in regions prone to frequent winds; water availability; a harvesting strategy and a physical route to market.

In EMF orchards, mycorrhiza monitoring of trees, usually from 2 y after planting, is strongly recommended (Baciarelli-Falini, Rubini, Riccioni, & Paolocci, 2006; De Miguel, Águeda, Sánchez, & Parladé, 2014) in order to obtain information on the persistence and development of the introduced fungal isolates (Guerin-Laguette, Cummings, Hesom-Williams, Butler, & Wang, 2013; Guerin-Laguette et al., 2014; Guerin-Laguette & Wang, 2015; Otsing & Tedersoo, 2015). Microscopy combined with molecular tools is the best approach. Microscopy can assess the actual development of the target species on the root system of host trees, such as the

presence of mycorrhiza clusters (Guerin-Laguette et al., 2013; Guerin-Laguette et al., 2014; Sánchez et al., 2014), and PCR- or sequence-based methods provides precision for the identification of some fungal species or isolates, sensitivity in the detection of extra-radical mycelium in soil (Parladé, De La Varga, De Miguel, Sáez, & Pera, 2013; Todesco et al., 2019), and a better understanding of the seasonal dynamics of extra-radical mycelia and ectomycorrhizae (Queralt, Parladé, Pera, & De Miguel, 2017). Combined, microscopy and molecular techniques also allow evaluation of the pressure of contamination from non-target mycorrhizal species. A recent study based on molecular techniques showed that a *Quercus ilex* L. forest surrounding 5-y-old *T. melanosporum*-inoculated *Q. ilex* trees increased the relative abundance of non-*T. melanosporum* mycorrhizal fungi in the soil of these trees when they were close to the forest without impairing the growth of the introduced truffle mycelium (Oliach et al., 2020). In an intensive EMF tree orchard, all trees are expected to be colonized by the desired EMF species. In large orchards, a statistical approach could be taken to estimate the number of trees that successfully host the introduced EMF species. With the persistence and development of the target EMF species established, it is likely that the orchard will produce fruiting bodies and, thus, is worth maintaining and managing.

5. Onset of fruiting

The onset of fruiting means the first production of edible basidiomata or ascomata of the target EMF species following controlled mycorrhization. This is the stage that most EMF cultivation trials reach, e.g. repeated fruiting following controlled mycorrhization without follow-up studies monitoring and documenting fruiting (yields) in orchards over time (e.g. cultivation *sensu stricto*). From the initial fruiting success, two approaches can be considered to further explore cultivation: controlled conditions (growth chamber, glasshouse) or field conditions.

Prior research has shown that well-mycorrhized seedlings can start producing fruiting bodies of their associated fungi under laboratory (growth chamber) or nursery conditions (Godbout & Fortin, 1990; Danell & Camacho, 1997; Villegas-Oliveira et al., 2017; Ogawa et al., 2019b), in some cases as early as 1 y or less after inoculation (Guerin-Laguette et al., 2000a; Yamada et al., 2001a). This includes the prestigious Périgord black truffle successfully cultivated by Albert Verlhac 2 to 3 y after planting mycorrhizal *Quercus pubescens* Willd. in ≈70 L containers in soil amended with micro-nutrients (e.g. boron, sulphur, iron) under a shade house (Sourzat, 1990). These observations suggest that the commercial production of edible mycorrhizal fungi could be explored in non-field, controlled environments such as glasshouses. The shape and size of the containers holding the host plants, the artificial substrates and nutrients, the environmental conditions could all be optimized to maximize fruiting-body formation. However, this line of research is virtually unexplored so far and would represent a significant investment of time and resources without guaranteed practical or commercial success.

To date, most advanced results in terms of EMF cultivation have resulted from fruiting in field conditions (i.e. out-planting mycorrhizal seedlings to establish productive tree orchards) following the pioneering work by Chevalier & Grente (1978) and Poitou, Mamoun, Ducamp, & Delmas (1984). Although this method has proven its efficacy for several species, very little is known or understood regarding the yield potential of EMF tree orchards. Truffles are no exception: there are very few studies aimed at documenting, understanding and optimizing the yields of truffle trees (Linde & Selmes, 2012; Reyna & Garcia-Barreda, 2014). Molinier et al. (2013) is one

of the rare studies documenting truffle yields over 35 y after planting. Yields of *T. aestivum* in France could exceed 300 kg/ha 13–15 y after planting, but not every year, (Chevalier, 2010; Todesco et al., 2019), while those of *T. melanosporum* are usually less, at about 100 kg/ha 15 y after planting (Molinier et al., 2013; Chevalier, personal communication, Aug 19, 2019). Some orchards in Western Australia are exceptional by world standards (500 kg/ha, G. Chevalier, personal communication, Aug 19, 2019). The reasons for their high production remains to be explained but recent findings showing fewer competing mycorrhizal fungi and a lower relative abundance of them in Australian than in European soils are consistent with the competition release hypothesis (Benucci et al., 2019). Increasing the knowledge on yields can strongly enhance the development of an EMF cultivation industry worldwide. Yield studies (per tree) looking at the impact of various factors such as time since planting, tree species, tree pruning, irrigation, genetic material, etc. are therefore urgently required in order to optimize production and allow the development of sustainable and economically viable EMF orchards.

6. Yield research (towards performing tree orchards)

Although obtaining fruiting bodies of a given EMF species in the field following controlled mycorrhization is a success in itself, this is only the start of the actual EMF cultivation research aimed at producing, reliably and sustainably, marketable quantities of fruiting-bodies in tree orchards. The next stage is therefore focused on determining how the tree orchard (established with mycorrhizal seedlings) must be designed and managed in order to optimize yields of that particular EMF species. As mentioned previously, in comparison with the cultivation of other horticultural products, especially cereals and fruit trees, the cultivation of EMF is still in its infancy. Significant research effort involving replicated treatments in full-scale experimental orchards is required before the yields of EMF trees can be increased and guaranteed. This will also inform understanding of other economically critical aspects associated with EMF production such as the lifespan of orchards. Apart from limited preliminary work on the mycorrhization of cloned hazels by *T. melanosporum* (Mamoun & Olivier, 1996) none of the genetic aspects, i.e. breeding for the most efficient plant/fungus partners, have been investigated so far.

There is a recent example of the yield potential of tree orchards established with mycorrhizal seedlings. Two saffron milk cap orchards were established at PFR-Lincoln in New Zealand in Dec 2007. The first was established with 42 seedlings of *P. radiata* (three trees died) and the other with 10 *Pinus sylvestris* L. (Scots pine) seedlings (all lived). The orchards are located 400 m apart, have a similar tree spacing, i.e. 4 × 5 and 5 × 5 m, respectively, which represent a density of 500 and 400 trees per ha, and have had the same irrigation regime (see also Guerin-Laguette et al., 2014; Guerin-Laguette, Butler, & Wang, 2020). The orchard layout and the history of mushroom production per tree, including the latest three fruiting seasons (2017–2019), are given in Figures 4 and 5 for the *P. radiata* and *P. sylvestris* orchards, respectively, thus covering 11 y since trial establishment. Table 1 and Figure 6 show the evolution of the total mushroom yields (kg per ha) for both orchards.
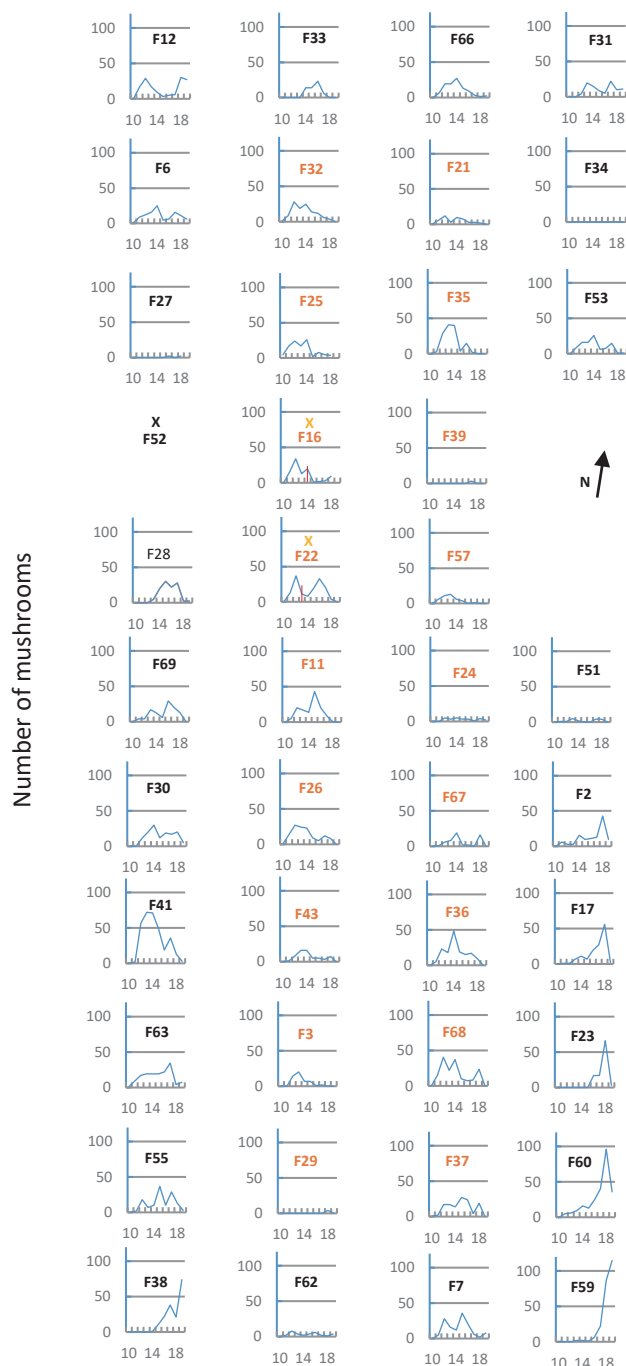
The two pine species have very different growth rates. Over the first nine y of the orchards, *P. sylvestris* seedlings growth reached only about a third of that of *P. radiata* (Guerin-Laguette et al., 2020). The difference in growth is still apparent in 2019 (Fig. 7). In 2015, canopy closure started in the *P. radiata* orchard with needles rapidly covering most of the inside of the orchard (Fig. 7B), while,

as of 2019, the *P. sylvestris* orchard has yet to reach this stage and still shows a grassy lawn around each tree (Fig. 7E). The sixth fruiting season of the *P. radiata* orchard (2015) marked a strong reduction of mushroom yields by over 50% compared to 2014 production, while the yields of *P. sylvestris* kept increasing until the sixth fruiting season reaching over 1000 kg per ha (Table 1; Fig. 6). Although the *P. sylvestris* orchard did not maintain such a high yield during the following two seasons (2018 and 2019), it still produced an average of ~730 kg/ha of mushrooms compared to less than 200 kg/ha for the *P. radiata* orchard during the same seasons, i.e. seventh and eighth fruiting seasons of the *P. radiata* orchard, 2016 and 2017 (Table 1; Fig. 6).

This study shows that host tree species can have a strong impact on the yield of an EMF orchard. The slower-growing *P. sylvestris* required a longer time than *P. radiata* to start producing mushrooms, i.e. 4 y following planting in our conditions (Fig. 6). However, the yields under *P. sylvestris* quickly increased and began to surpass those of *P. radiata* from 2015 onward (Table 1; Fig. 6). Therefore, under the field conditions tested, *P. sylvestris* proves to be a highly efficient host pine species to cultivate saffron milk caps. In contrast, *P. radiata* gave quicker results, i.e. 2 y after planting, but yields dropped considerably from the sixth fruiting season, at least under the management regime (no pruning) used in the present study. The high yields observed in these *L. deliciosus* orchards in New Zealand may also be partly explained by the 'competition release' hypothesis, e.g. fewer ectomycorrhizal fungi competing with these two species in their land of adoption in comparison with the level of competition that they would experience in their native habitat (Europe). As exposed previously (Section 5), a similar hypothesis is mentioned for exceptionally high *T. melanosporum* yields in Australia (Benucci et al., 2019). However, under optimal rainfall, yields over 1000 kg per ha have also been obtained for the desert truffle *T. claveryi* in an orchard established in its natural area of distribution (Morte, Andrino, Honrubia, & Navarro Rodénas, 2012).

Saffron milk cap mushrooms produced in 2018 and 2019 seasons are shown on Figure 8. Canopy closure in the *P. radiata* orchard represents a turning point in the production of saffron milk caps, potentially caused by several factors such as: decrease in light and soil temperature inside the orchard, competition between the fungus and the host tree for water, as also postulated for *T. melanosporum* by Büntgen et al. (2012), and disappearance (Fig. 7D) of the grass cover that provides an ideal micro-habitat habitat for saffron milk cap fruiting (Guerin-Laguette et al., 2020). From 2015, production data in the *P. radiata* orchard show that mushroom fruiting progressively occurs on trees located on the edge of the orchard and almost exclusively on those trees in 2019 (Figs. 4, 9). A few mushrooms growing underneath the needle litter inside the *P. radiata* orchard may have been missed but are unlikely to represent a significant biomass. We carefully looked for litter mounds suggesting the presence of mushrooms and while some revealed saffron milk caps (Figs. 7D, 8E), most were due to other mushroom species (Guerin-Laguette et al., 2014). The most productive trees to date produced 115 and 226 saffron milk cap mushrooms in 2019 under *P. radiata* and *P. sylvestris*, respectively (Figs. 4, 5).
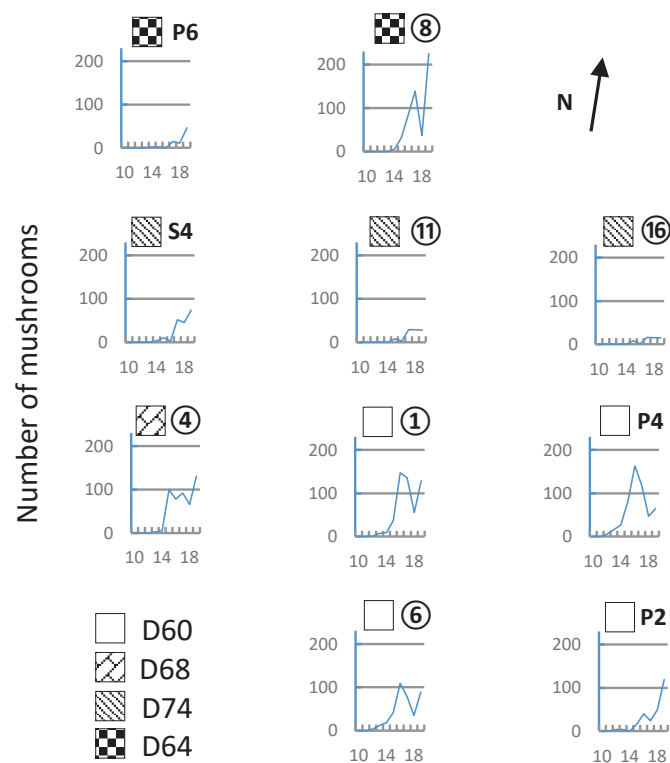
From the yield data presented here, several hypotheses for future field trials on saffron milk cap cultivation can be formulated. Could the pruning of *P. radiata* before canopy closure halt the decline in mushroom production? If so, what would be the yields of pruned *P. radiata* trees in comparison with those of unpruned ones? Could the yields of pruned *P. radiata* be as high as, or even higher than those observed on *P. sylvestris* before canopy closure during this study? Canopy closure in the *P. sylvestris* orchard is ex-

Harvest years: 2010 to 2019

Fig. 4. – Layout of the *Pinus radiata* orchard (see also Guerin-Laguette et al., 2014, 2020) and history of mushroom production for each tree. As of 2020, the orchard is composed of 39 trees over ≈ 1,000 m². Each F number locates a *P. radiata* tree. The graphs indicate the numbers of fruiting bodies harvested from each tree from 2010 to 2019, except for trees F27, 25 and 16 where no mushroom number could be assigned to these trees in 2019. Abundant fructification (96 mushrooms) was found, mostly at the south of tree F27, spread in the grassy patch left by the disappearance of nearby trees (F52, 16 and 22). This could correspond to underground *Lactarius deliciosus* mycelium being mycorrhizal with the root systems of nearby standing trees. This fructification was counted as coming from edge trees in 2019. Trees in orange are considered 'inside' in comparison with those in black that are on the edge of the orchard (see also Fig. 9). A cross indicates a dead tree: Tree F52 died during the first year after planting while trees F22 and F16 were lost to wind storms in 2013 and 2014, respectively (vertical red bars mark the death).

pected to also reduce mushroom yields. Could high yields obtained under *P. sylvestris* prior to canopy closure be maintained by pruning the trees and, if so, for how long? Of course, the method and the intensity of tree pruning would also need to be tested. Full-scale replicated field trials with *P. sylvestris* and *P. radiata* monitoring mushroom yields in function of the tree pruning regime could answer these questions and further improve the efficiency of saffron milk cap cultivation. Finally, thinning could also be a way to invig-

Harvest years: 2012 to 2019

Fig. 5. – Layout of the *Pinus sylvestris* (Scots pine) orchard and history of mushroom production for each tree. The orchard is composed of 10 trees over 250 m². Each square indicates a *P. sylvestris* tree inoculated with a distinct *Lactarius deliciosus* isolate, i.e. D60, D68, D74 or D64 (see also Guerin-Laguette et al., 2020). The graphs indicate the numbers of fruiting bodies harvested from each tree from 2012 to 2019.
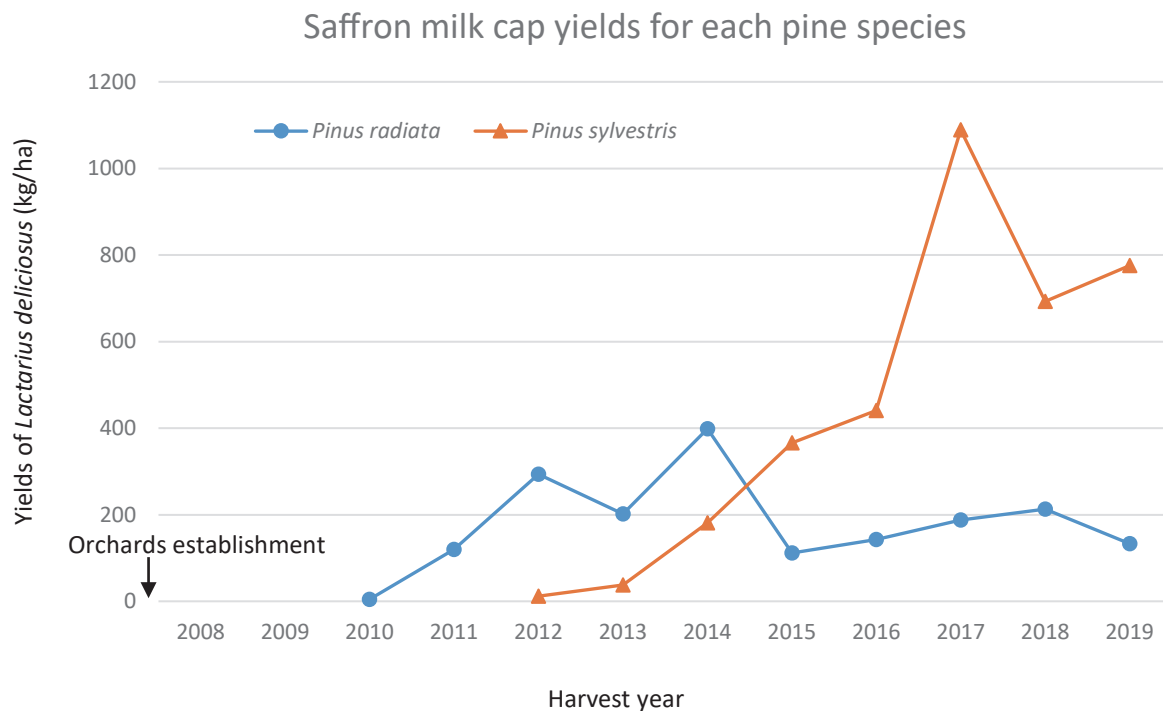
Table 1. Yield of saffron milk cap *(Lactarius deliciosus)* mushrooms harvested under *L. deliciosus* mycorrhizal seedlings of *Pinus radiata* or *P. sylvestris* out-planted on the Lincoln campus of The New Zealand Institute for Plant & Food Research Ltd in Dec 2007.

Year	Fruiting season	Yield[a] (kg per ha)	Fruiting season	Yield[a] (kg per ha)
		Pinus radiata		*Pinus sylvestris*
2008	-	-	-	-
2009	-	-	-	-
2010	1st	5	-	-
2011	2nd	120	-	-
2012	3rd	294	1st	12
2013	4th	202	2nd	38
2014	5th	399	3rd	181
2015	6th	112	4th	366
2016	7th	143	5th	441
2017	8th	188	6th	1,089
2018	9th	213	7th	693
2019	10th	133	8th	776

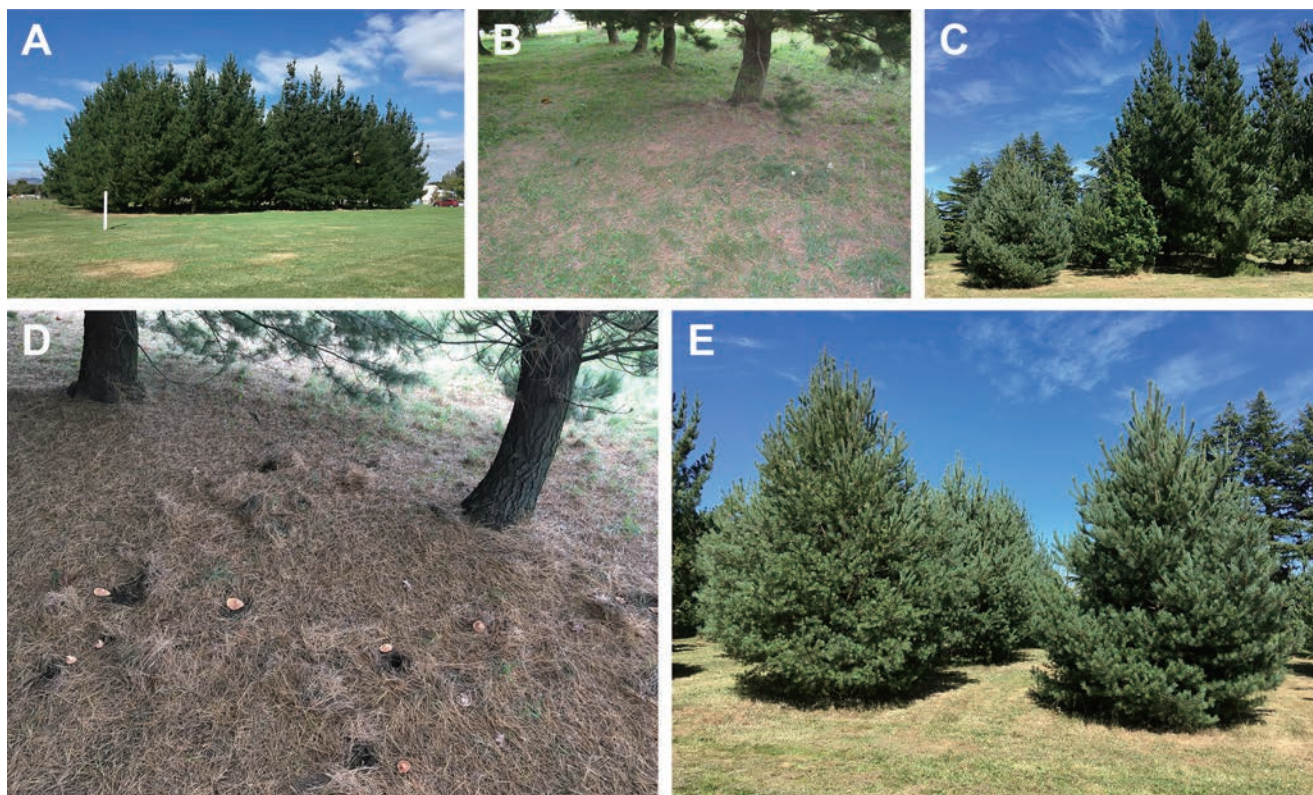[a] Sum of fresh weight of cleaned mushrooms (without debris) measured at each harvest day over the full season.

- Not applicable

Saffron milk cap yields for each pine species

Fig. 6. – Yields (kg/ha) of *Lactarius deliciosus* (saffron milk caps) produced by the *Pinus radiata* or *P. sylvestris* orchard over ten and eight production years, respectively. Orchards were established in Dec 2007.

Fig. 7. – Views of the *Pinus radiata* and *P. sylvestris* orchards including grass cover or pine needle litter around trees. A: View of the *P. radiata* orchard (west side) and its canopy closure on 19 Mar 2019; B: The needle deposition layer is apparent inside the *P. radiata* orchard on 11 May 2015; C: Comparative view on 22 Jan 2019 of the growth of trees of the saffron milk cap x *P. sylvestris* orchard (left) versus that of *Boletus edulis* x *P. radiata* trees (right), all planted in 2007; D: Thick needle litter inside the *P. radiata* orchard (from left, trees F23 and F60) with mushrooms growing underneath the litter around tree F60 on 15 May 2019; E: Partial view of the east side of the *P. sylvestris* orchard (from left, trees P4 and ⑯) on 22 Jan 2019 showing the lawn cover that can still develop around trees 11 y after plantation because of the lack of canopy closure.
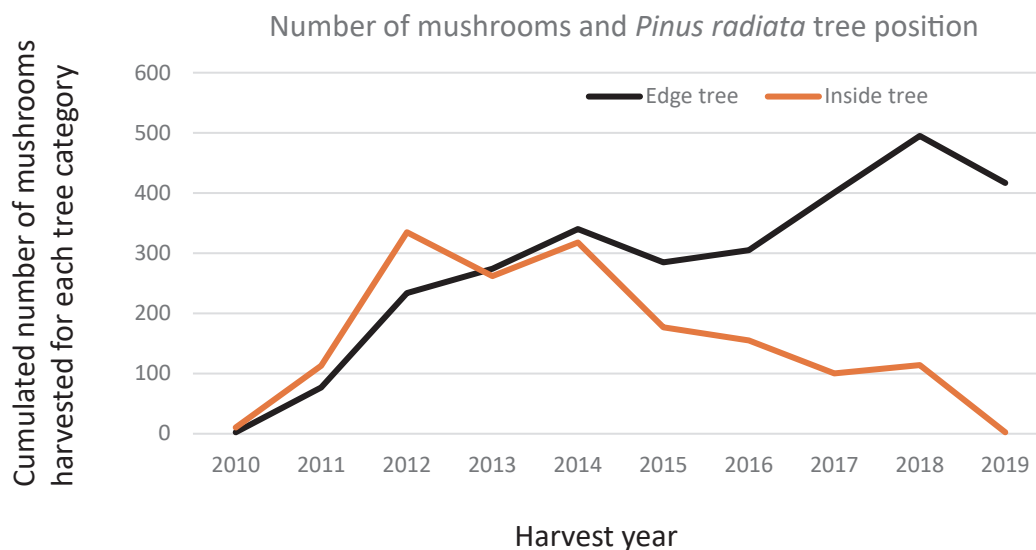
Fig. 8. – Fruiting and harvests of saffron milk cap (*Lactarius deliciosus*) under two pine species. A–C: Morphological characteristics of *L. deliciosus* basidiomata. A: Concentric circles on the cap (*Pinus radiata*, 29 Jan 2018). B: Abundant milk coming off the stipe of freshly harvested fruiting-body (*P. radiata*, 2 Mar 2018). C: 'Scrobicules' or orange stains on the stipe (*P. sylvestris*, 30 Apr 2019). D–F: *P. radiata* site. D: Grass cover and mushroom production on the edge of the orchard, south of tree F59, 30 Apr 2019. E: Mushroom production inside the orchard under the pine needle litter close to the trunk, southwest of tree F60, 30 Apr 2019. F: One day-harvest under tree 60, 5 March 2018. G–I: *P. sylvestris* site. G: Harvest from several trees on 3 May 2019. H: High density of fructification around tree ⑧ (75 mushrooms harvested around that tree on 3 May 2019). I: Example of fructifications in the needle litter below the canopy of *P. sylvestris*, 3 May 2019.

orate mushroom yields in ageing forest stands (Egli, Ayer, Peter, Eilmann, & Rigling, 2010).

Like for any other outdoor crop, the effect of climate on the inter-annual variability of the yield of cultivated or naturally fruiting edible fungi is strong and well documented in the literature. Several studies have shown how climatic variables impact the yield of European black truffle species (Büntgen et al., 2012; Le Tacon et al., 2014; Molinier et al., 2013). At the orchard level, Molinier et al. (2013) have shown strong positive correlations between tempera-

tures in Aug (maximum, minimum and average) and the number and total weight of *T. melanosporum* truffles harvested. The orchard was not irrigated and the authors also showed that temperature/cumulative rainfalls in May and Oct positively correlated with the average truffle weight. At the regional level, Büntgen et al. (2012) found that French and Spanish *T. melanosporum* harvests showed significant positive correlation with summer rainfall. In France, Le Tacon et al. (2014) observed that the cumulative hydric balance from May to Aug of the year *n* was the main factor explain-

Fig. 9. – Evolution of the number of mushrooms produced by trees according to their position in the *Pinus radiata* orchard, i.e. 'edge trees' versus 'inside trees' (see Fig. 4), every year since the start of fruiting in 2010. Canopy closure and needle deposition started approximately in 2015.

ing the large annual variations in the total weight of truffle sold in the two main south eastern wholesale markets of the Périgord black truffle. Andrino, Navarro-Rodénas, Marqués-Gálvez, & Morte (2019) showed that the crop of the desert truffle *Terfezia claveryi* depends on agroclimatic parameters during two key annual periods (autumn: Sep to Oct and spring: end of Mar) and that the aridity index and the soil water potential could be easily controlled during these periods by applying irrigation. Scientists are also developing new markers such as the phenology status of the host plant (*H. almeriense*) during the year to explain or manage desert truffle production (Marqués-Gálvez, Morte, & Navarro-Rodénas, 2020). For cultivated pine orchards in the southern hemisphere, Guerin-Laguette et al. (2020) have shown the impact of summer and autumn rainfall on triggering major peaks of saffron milk cap mushroom fruiting, including an increase in the average mushroom weight. These authors have also shown that soil temperatures warmer than usual were involved with a rare winter fruiting of the saffron milk cap in Jul 2017. In a continental Mediterranean climate in Spain, the positive impact of autumn rainfall and temperature (i.e. wetter and warmer than normal) on yields of naturally occurring *Lactarius* sect. *Deliciosi* and *Boletus edulis* mushrooms was evident over a 15 y study in pure even-aged *P. sylvestris* stands (Martínez-Peña et al., 2012). Finally, a recent Spanish study highlights the potential of remote sensing to acquire both climatic and plant (primary productivity) data at a regional level (Olano et al., 2020). In combination with traditional fixed climatic station data, this approach could improve both mushroom yield models and our understanding of the environmental factors driving edible fungi production.

Apart from the study by Molinier et al. (2013), there is almost no other work describing, continuously over time, the yield of truffle or mushroom orchards established with seedlings mycorrhized under controlled conditions. Further, to the best of my knowledge, the yield per tree over time has almost never been monitored and reported in scientific studies. The saffron milk cap orchards presented here highlight the importance of conducting yield monitoring research, tree by tree, over time, and in function of management treatments in order to increase our understanding of the potential yields that EMF commercial orchards could achieve in the future. However, after several years, e.g. 7 y after planting in the

case of *P. radiata*/*L. deliciosus*, the root systems of neighbouring trees begin to overlap (i.e. there is an underground network of mycelium and roots) and it becomes difficult to assign fruiting-bodies to particular trees with certainty (Guerin-Laguette et al., 2020). For desert truffles, the host plants are mycorrhizal shrubs that may be arranged closely in groups (Morte et al., 2017) making it almost impossible to assign ascomata to a given shrub, especially since fruiting can occur several centimetres or meters away from the shrubs. Although it is desirable to monitor the yield per host plant whenever possible, this approach may not be suited to certain EMF species or will need to be replaced with yield per surface unit as the orchards mature.

7. Future challenges and opportunities

7.1. Cultivable species

Cultivable species are EMF species for which controlled mycorrhizal synthesis is mastered and followed by fruiting-body production after transplantation of mycorrhizal seedlings in suitable soil. The best examples of cultivable species are Ascomycete truffle species, *Tuber* e.g. *T. melanosporum*, *T. borchii*, *T. aestivum*, *T. indicum* Cooke & Massee, or the desert truffles *Terfezia claveryi* Chatin, first cultivated with *Helianthemum almeriense* Pau in Spain in 2001 (Morte, Honrubia, & Gutiérrez, 2008) and *Terfezia boudieri* Chatin, cultivated with *Helianthemum sessiliflorum* (Desf.) Pers. in Tunisia in 2006 (Slama et al., 2010) and in Israel (Sitrit Y., unpublished; A. Morte, personal communication, Jun 4, 2020). Other species of desert truffles, *Picoa lefebvrei* (Pat.) Maire and *Terfezia crassiverrucosa* Zitouni-Haouar, G. Moreno, Manjón, Fortas, & Carlavilla, were recently cultivated with *H. almeriense* in 2020 in Spain (A. Morte, personal communication, Jun 4, 2020). Only a few Basidiomycete mushroom species, e.g. *L. deliciosus*, *S. luteus*, and *R. rubescens* have been cultivated in tree orchards.

Statistically robust field trials incorporating key replicated treatments such as tree pruning, tree species and irrigation regime are required in order to measure EMF production and understand how these treatments impact yields. These trials would generate the knowledge required to sustain high yields, and could potentially make future commercial EMF orchards more productive. In the

case of truffles, the cultivation system is more complex (Le Tacon et al., 2016). Shallow soil aeration and superficial trimming of mycorrhizal roots would be important treatments to add, as would be spore additions to the soil in the case of *T. melanosporum* (Murat et al., 2016) and other truffle species. The monitoring of the truffle yield itself is more challenging than for epigeous mushrooms since it relies on the use of trained and efficient dogs. However, the high commercial value of truffles could justify a higher investment in yield research.

7.2. Recalcitrant species

Recalcitrant species are EMF species for which mycorrhization technology is still challenging or expensive, e.g. *T. magnatum*, *Cantharellus* spp., *T. matsutake*, *B. edulis* s.l., and which, to the best of my knowledge, has yet to be followed by fruiting-body production after plantation of mycorrhizal seedlings in the field, e.g. *T. matsutake* (Yamanaka, Yamada, & Furukawa, 2020).

Improving mycorrhization technology could be one way to unlock their cultivation. Recently, Horimai et al. (2020) have shown that multiple inoculation of genetically different isolates of *T. matsutake* has led to enhanced mycorrhization of *P. densiflora* in comparison with single isolate inoculation. Other examples are probably the Japanese yellow chanterelle (*Cantharellus anzutake* W. Ogawa, N. Endo, M. Fukuda & A. Yamada) and the prestigious Italian white truffle (*T. magnatum*), for which recent advances have been made. For example, Ogawa et al. (2019a) developed an efficient method to isolate pure cultures of the Japanese yellow chanterelle from ectomycorrhizal root tips. This was an important breakthrough since pure cultures of edible chanterelles are difficult to obtain from basidiomata tissues (Danell, 1994). This method provided access to pure culture isolates from which mycorrhizae were further obtained under *in vitro* conditions. The successful acclimation of mycorrhizal seedlings under clean, non-sterile growth chamber conditions, and the progressive increase of the size of the containers (up to 4 L) led to the repeated fruiting of this species over two y at 20–24 °C under continuous light illumination (Ogawa et al., 2019b). This is a significant achievement since fruiting of true chanterelles has only been reported once in the past, i.e. the first ever cultivation of the golden chanterelle (*C. cibarius*) in Sweden by Danell & Camacho (1997). However, the early success with *C. cibarius* was not followed by successful commercial applications or further research. In Japan, ongoing trials with *C. anzutake* now involve the monitoring of out-planted mycorrhizal seedlings (A. Yamada, personal communication, Feb 20, 2020).

As far as *T. magnatum* is concerned, a patent (EP2268790A1) covers the isolation and pure culture of *T. magnatum* mycelium from ascoma tissue and, since 2010, a confidential agreement between the French National Institute for Agricultural Research (INRA) and a private nursery (Robin Pépinières EARL, France) allows for the commercial production of seedlings mycorrhizal with *T. magnatum*. Each seedling is tested with DNA-based methods to ensure the presence of *T. magnatum* within the host root tissue (C. Murat, personal communication, Jul 24, 2019). Monitoring of seedlings with DNA approaches confirmed the presence of *T. magnatum* in tree orchard soils 8 years after they were established and planted in the field (Robin et al., 2019). These results are encouraging but, to date, no truffles have been produced from these seedlings, thus *T. magnatum* still defies cultivation.

7.3. Innovation: orchards with multiple EMF species

Under the current model, EMF tree orchards are established with a single target species. The co-existence of several mycorrhizal species on trees in nature (Bahram, Põlme, Kõljalg, & Tedersoo, 2011; Gehring, Theimer, Whitham, & Keim, 1998) suggests that this could be considered as an avenue for increasing the number of edible species produced in one orchard. The best example comes from the association between pines and several EMF species: *Tuber* spp. and *Lactarius* sect. *Deliciosi*. The fruiting of *L. deliciosus* in PFR's *T. borchii* orchard (see section 2) highlights the feasibility of this approach. However, *Lactarius sanguifluus* (Paulet) Fr., a thermophilic (predominantly Mediterranean) milk cap species adapted to calcareous or calcic soil horizons saturated with calcium ions (Ca^{2+}) and having neutral or high pH (D. Mousain, personal communication, Jun 11, 2020) is highly regarded as an edible mushroom (Borgarino & Hurtado, 2001) and could be therefore an ideal autumn companion of *T. borchii* or *T. aestivum* pine orchards. This dual culture would offer the double advantage of (1) potentially reducing the pressure of contamination by foreign ectomycorrhizal fungi (as the more of the introduced species of interest on the tree root systems, the less room for non-target ectomycorrhizal species), and (2) diversifying and lengthening the production season, and therefore increasing the commercial benefit from the orchard. More research is required to explore this concept, such as techniques for producing plants co-inoculated by two or more EMF species or chequerboard planting of single-EMF species seedlings in the hope that the target EMF species will gradually propagate over all the orchard.

8. Conclusions and long-term benefits

International trade in edible wild mushrooms, most of which are EMF, has considerably increased recently (de Frutos, 2020) and the long-term potential of cultivating and managing EMF is promising. Significant research investments are required to improve EMF cultivation. The present work on saffron milk cap cultivation indicates that it is possible to obtain high EMF yield from managed tree orchards. Establishing full-scale experimental EMF orchards, in which management treatments are tested statistically, is one priority for future research, as are an improved understanding of fruiting mechanisms of EMF (Le Tacon et al., 2016; Ogawa et al., 2019b), genomic and genetic studies (Martin & Bonito, 2012) aimed at elucidating fruiting mechanisms, and selecting or breeding for efficient symbionts (Saito et al., 2018; Yamada et al., 2019). The saffron milk cap yield data presented in this review can also serve as a reference for future full-scale replicated trials.

Beyond producing food and providing economic benefits, EMF cultivation has the potential to contribute to some of today's major challenges for human societies. For example, EMF cultivation could contribute towards global efforts to counter emission levels since C-fixing perennial trees are required to cultivate EMF. Pruning wastes from EMF orchards could provide a renewable source of bioenergy (Hevia, Crabifosse, Álvarez-González, Ruiz-González, & Majada, 2017) or organic matter to improve soil. EMF could also contribute to diversify the sources of non-meat protein in human diets, as EMF fungi are rich in amino acids, minerals and vitamins (Muszyńska et al., 2016). EMF production systems can play a role in local food production since orchards could be established in relatively small farm land areas surrounding urban consumption centres, reducing transportation fuel costs. They could also contribute to environment and biodiversity protection by creating forested land that provides habitats for wildlife, including potential predators of pests of neighbouring crop fields, and supporting natural capital (England et al., 2020). Finally, EMF cultivation could provide opportunities to develop lifestyle blocks (New Zealand term

designing a semi-rural property comprising a house and land for small-scale farming), contributing to a green economy, myco-tourism and the wellbeing of communities (Benucci et al., 2012; Büntgen, Latorre, Egli, & Martínez-Peña, 2017; Martinez-Ibarra, Gómez-Martín, & Armesto-López, 2019).

There are avenues for further enhancing and diversifying the performance of EMF orchards. In the coming years it is likely that multiple EMF species will be cropped in one orchard (e.g. several mushroom and truffle species grown in the same piece of land), and that economic trees (e.g. pine nuts, pecan nuts, chestnuts) will be cropped in combination with edible fungi (Benucci et al., 2012; Álvarez-Lafuente, Benito-Matías, Peñuelas-Rubira, & Suz, 2018; Mei, Liu, Tang, Wang, & Guerin-Laguette, 2019). For all these reasons, it is important that the mycological community continues research aimed at domesticating these discrete but powerful delicacies.

Disclosure

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