Article

Fl. Medit. 32: 367-374
https://doi.org/10.7320/FlMedit32.367
Version of Record published online on 28 December 2022

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Use of *Suillus collinitus* to increase survival rate and resilience of transplanted *Pinus halepensis* seedlings in habitat restoration practices

Abstract

Iannaccone, M., Lamoliere, A. & Buhagiar, J. A: Use of *Suillus collinitus* to increase survival rate and resilience of transplanted *Pinus halepensis* seedlings in habitat restoration practices. — Fl. Medit. 32: 367-374. 2022. — ISSN: 1120-4052 printed, 2240-4538 online.

In-situ conservation is considered the best way to preserve biodiversity where multiple species can persist with all their natural ecological interactions. The latter element is often ignored. Criticalities can arise when *ex-situ* grown plants are translocated *in-situ* without their natural soil microbial associations, especially where abiotic stresses can be severe. Mycorrhizal Fungi (MF) provide several benefits to plants and ecosystem in which are present. The aim of the project was to investigate the use of indigenous MF to enhance acclimatization and survival of *ex-situ* grown plants translocated for habitat restoration. The seeds of *Pinus halepensis* Mill. as well as sporocarps of naturally associated indigenous MF *Suillus collinitus* (Fr.) Kuntze (1898), were collected from Natura 2000 sites in the Maltese Islands. *P. halepensis* seeds were germinated under greenhouse conditions. *S. collinitus* was inoculated at sowing and seedling stage by placing fragments of the ripe sporocarps in the *P. halepensis* seedling trays. All the mycorrhized plantlets produced have been reintroduced into selected area of Natura 2000 in the Maltese Islands as part of SiMaSeed restoration project. Such MF inoculation actions can help to enhance restoration and resilience of habitats to reduce biodiversity loss.

Key words: ectomycorrhizae, Mediterranean flora, habitat restoration.

Introduction

The UN's 2030 Agenda for Sustainable Development calls for the preservation of biodiversity, which is threatened due to many factors, among them habitat degradation (www.sdgs.un.org/goals). Actions to enhance restoration and resilience of habitats to reduce biodiversity loss are needed.

Though *in-situ* conservation is considered the best way to preserve biodiversity where species can persist with all their natural ecological interactions, the latter element is often ignored (Godefroid & al. 2011; Fenu & al. 2019). Criticalities can arise when *ex-situ* grown indigenous plants are translocated *in-situ*, without their natural soil microbial associations, especially in a Mediterranean climate where abiotic stresses are severe (Doyle & al. 2021).

Factors which affect plant acclimatization as well as possible solutions for a better establishment *in-situ* are summarised in Doyle & al. (2021). Among possible solutions, Haskins & Pence (2012) found that plant translocation is more successful if mutualistic relationship with soil microbiota have been established. Mutualistic relationship between mycorrhizal fungi and plant, promote plant productivity, alleviate abiotic and oxidative stresses, enhance access to micro- and macro- nutrients as well as water (Smith & Read 2008).

The thermophilous vegetation of the Maltese Islands, located in the middle of the Mediterranean Basin, has been extensively described in Sommier and Caruana Gatto (1915), Borg (1927), Haslam (1969), Haslam & al. (1977), Lanfranco (1984, 1995a), Schembri (1994, 1997), Lanfranco & Schembri (1986), Anderson & Schembri (1989), Savona-Venutra (2001), Casha (2015), Brullo & al. (2020).

At present, woody vegetation in the Maltese Island is restricted to localized areas, though, presumably in the past it was much more abundant as many toponyms might confirm (Brullo & al. 2020). For instance, Wied Żnuber which in Maltese translates to Aleppo pine valley, suggests that long ago it was populated by stands. Wied Żnuber is part of the Special Protected Area of Conservation of International Importance of Rdumijiet ta' Malta: Ir-Ramla tac-Cirkewwa sa Il-Ponta ta' Benghisa (MT 0000024). The above-mentioned valley currently records few individuals of *P.halepensis*.

In the Maltese Island the *P. halepensis* is only cultivated in gardens or street edges, as well as it is used for reforestation and has been found associated with the ectomycorrhizal fungus *Suillus collinitus* (Fr.) Kuntze (1898), by several authors including Lanfranco (1995b), Mifsud (2011), Iannaccone & Buhagiar (2020).

The aim of the project was to restore part of the population of *P. halepensis* present in Wied Żnuber with indigenous ectomycorrhizal fungus *S. collinitus.* This was intended to enhance acclimatization and survival of seedlings that had prior inoculation of *ex-situ* grown plants, post transplantation.

Material and Methods

Plant material

One-year-old *Pinus halepensis* cones were collected in August 2019 from an Aleppo pine stand in Għajn Tuffieħa and then stored at the Seed Bank of the Biology Department at the University of Malta. In November 2020 cones were opened at 80°C in a dry oven for 3 hours. Seeds were cleaned from bracts, then sown at the beginning of January 2021 in a mix of premium compost (Mikskaar), white peat (Mikskaar) and perlite (Kauf) (2:3:1) in square ribbed nursery pots (ARCA™, 8 × 8 × 12 cm). Seeds were sown 1 cm below the surface. A total of 10 replicates were prepared. Each replicate consisted of 6 pots, with 5 seeds per pot (N=30), for a total of 300 seeds. Pots were then watered by capillarity and exposed to Maltese natural environmental conditions in a semi-shaded area. After germination, seedlings were watered as required.

The seed were considered germinated after the full emergence of the cotyledons in erect position, since it was not possible to perform any observation of the radicle as commonly agreed for germination experiments (Come, 1970). Seedling emergence was recorded after a total period of 4 months under local natural conditions of temperature and humidity, after ambient temperatures rose above 15°C, as shown in Figure 1. After a period of 6 months, site was surveyed and the survival rate of non-mycorrhized plants as well as mycorrhized was assessed.

Fungal material

Four medium sized sporocarps of *S. collinitus* were collected from Il-Buskett, limits of Dingli, Malta in December 2020, cleaned under running water, chopped, and blended in 2 litres of sterile distilled water and stored at 4°C. Two inoculations were performed, the first a week after sowing *P. halepensis* seeds, the second on 8 weeks-old *P. halepensis* seedlings. The inoculation was performed by diluting 1 litre of the mother solution into 3 litres of sterile distilled water, then pots were soaked in such solution until the substrate was saturated (Chen & al. 2006). Mycorrhization was visually assessed by removing the seedlings from the pot and examining the root morphology as well as the presence of the mycelium (Chen & al. 2006).

Before and after being granted approval from the Environment & Resource Authority (ERA) as the responsible body, several surveys were carried out before proceeding with the planting intervention in order to identify the most suitable area for transplantation,

Results and Discussion

Pinus halepensis Mill.

Malta: Għajn Tuffieħa, Mellieha, Malta, (WGS84: 35.931195°N, 14.344549°E), J.A. Buhagiar, M. Iannaccone and A. Lamoliere (SiMaSeed/BDUM/19/93, Seedbank of the Department of Biology, University of Malta).

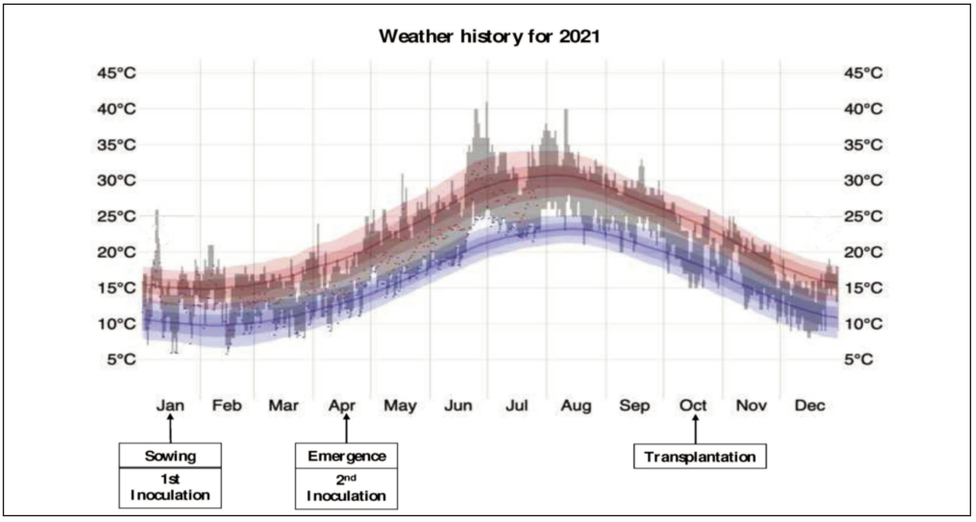

Fig. 1. The daily range of reported temperatures (grey bars) and 24-hour highs (red ticks) and lows (blue ticks), placed over the daily average high (faint red line) and low (faint blue line) temperature, with 25th to 75th and 10th to 90th percentile bands (Wheaterparks modified by M.Iannaccone).

Sample size: 300 seeds

The total germination under seminatural condition was of 52%. The present results agreed with Thanos (2000). As expected, due to 19 months of storage in a semi-controlled environment, seed viability has generally decreased compared to other results mentioned in Nunes & Calvo (1999), Thanos (2000), Daskalakou & Thanos (2004) as well as references within.

Suillus collinitus (Fr.) Kuntze (1898)

Malta: Il-Buskett, Ħad-Dingli, Malta, **(**WGS84: 35.859490°N, 14.397619°E), J.A. Buhagiar, M. Iannaccone and A. Lamoliere (MYCOBDUM-MI/2020/1), Mycobank of the Department of Biology, University of Malta).

The ectomycorrhizal fungus *Suillus collinitus* has been commonly found associated with *P.halepensis* (Galli 2013). Collection date November 2020. As the source of inoculum spores, as well as mycelium, have been widely used by Cordell & al. (1987), Torres & Honrubia (1994), Honrubia & al. (1997), and González-Ochoa & al. (2002) to achieve mycorrhization. The use of mycorrhized plants for afforestation has been studied by Le Tacon & al., 1988 and in the specific case of *P. halepensis* and *Pisolithus arhizus* (Scop.: Pers.) Rauschert 1959 by Querejeta & al. (1998).

In our project, a total of 59 seedlings of *P. halepensis* were translocated *in-situ*, of which 11 were successfully mycorrhized with *S. collinitus,* as shown in Fig. 2. The remaining 48 did not shown any evidence of mycorrhization. It was observed that the growth performance of mycorrhized *P. halepensis* in the nursery, survived periods of summer heatwaves better

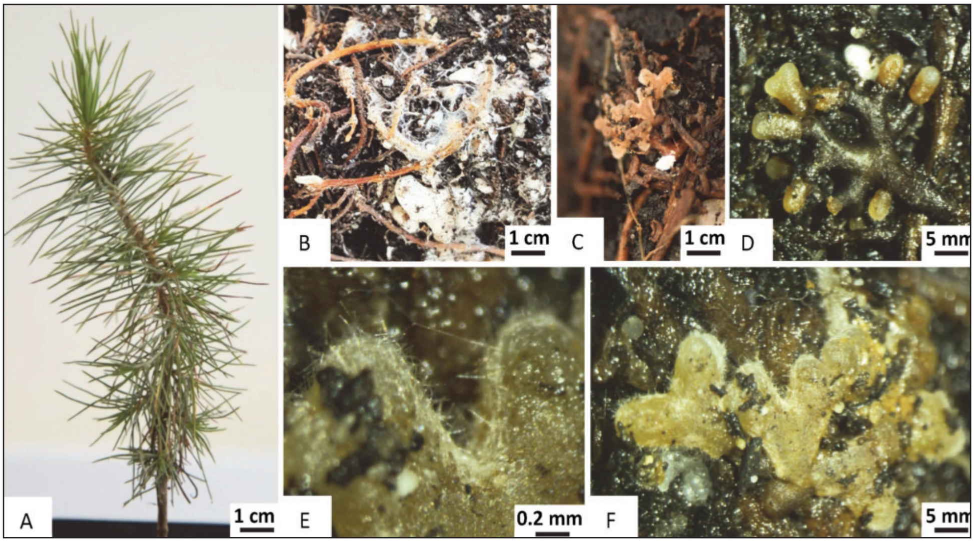

Fig. 2. **A.** *Pinus halepensis* seedling. **B.** *P. halepensis* root system embedded in *Suillus collinitus* mycelium. **C-D.** *P. halepensis* modified roots associated with *S. collinitus*. **E-F.** Close up.

than non-mycorrhized seedlings (Smith & Read, 2008). The same observation is valid for *in-situ* transplanted seedlings after a period of 6 month, as shown in Fig. 3.

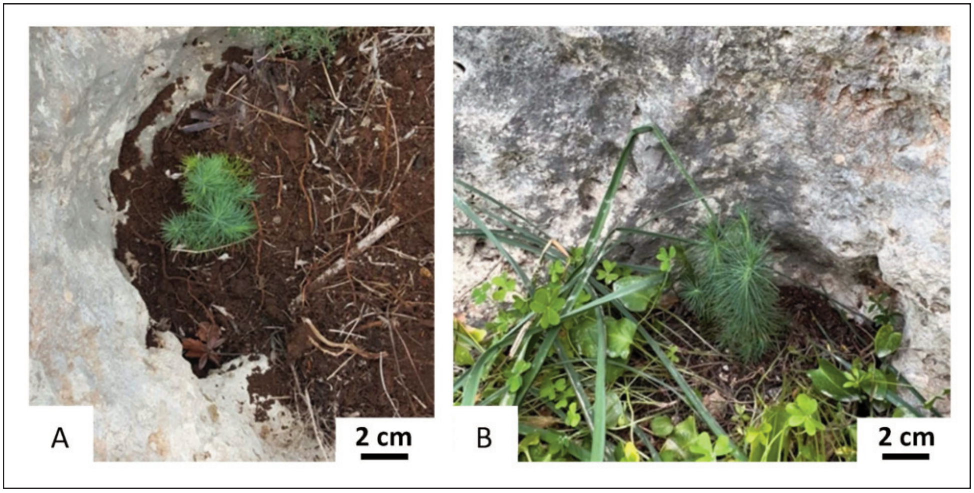

Fig. 3. *Pinus halepensis* associated with *S. collinitus* **A.** Translocated. **B.** 6-months after translocation.

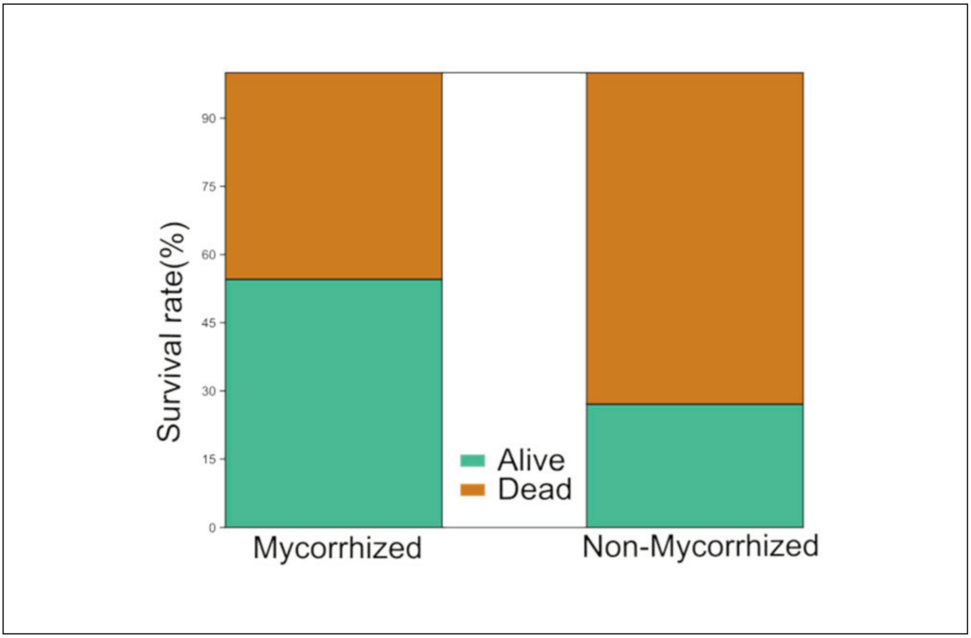

Fig. 4. Difference in survival rate (%) between Mycorrhized and Non-Mycorrhized plants.

As regards survival rates of transplanted plants after a period of six months, it was observed that the non-mycorrhized seedlings have higher mortality compared with the mycorrhized seedlings. Though no further examination on dead and surviving seedlings were carried out, the mycorrhized seedlings probably benefited from the advantages provided by the ectomycorrhizae association of *S. collinitus* and also other biotic and abiotic factors.

For further studies, a larger experimental sample should be taken into consideration, whilst the subjects from the present study will continue to be monitored. Furthermore, it is suggested that the use of indigenous arbuscular mycorrhizal fungi as well as other soil-born beneficial fungi such as *Trichoderma* spp. be given more importance in habitat restoration practices.

Conclusions

Through the present research, it was possible to confirm that cones of *P. halepensis* can be preserved in semi-controlled conditions for at least 19 months, whilst still maintaining a good degree of viability at 52% seed germination.

It was also possible to successfully associate ectomycorrhizal fungi *S. collinitus* to *P. halepensis* simply by introducing liquid inoculum containing spores and mycelium. Furthermore, such association appear to enhance plant acclimatization in *in-situ* translocation.

Acknowledgements

This work was supported by the SiMaSeed Project "Protecting biodiversity in Sicily-Malta Natura 2000 sites through Seed Banks and population reinforcement." Programme INTERREG V-A Italia-Malta 2014-2020. Priority Axis III, Specific Objective 3.1, Project code C1-3.1-16.

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