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AGRICULTURAL UNIVERSITY OF ATHENS

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"Conservation of priority species and habitats of Andros Island protected area integrating socioeconomic considerations"

ACTION C.2

Final Report on the alder seeds, the fungal (ECM) inocula and the alder seedlings produced for the restoration of alluvial forest in priority habitat 91E0*

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Abstract

Alder stands of Andros island have suffered extensive damages during the past few years which also had a negative impact on the symbiotic microbial structure of their habitat, thus making their natural regeneration very difficult. At these sites, re-establishment of the population of *A. glutinosa* was decided to be performed through targeted planting of alder seedlings colonized with their symbiotic microorganisms in order to enhance their establishment and long-term survival. Several field-trips and various activities took place during the period 2017-2019, which were associated to alder seeds and fungal inocula collection, establishment of fungal inocula, inoculation trials, seeds germination, seedlings development and their inoculation, and alder seedlings transplanting to the restoration areas. Adequate amount of alder seeds was collected during two consecutive seasons (late autumn of 2017 and 2018), were stratified to overcome dormancy and sowed at large numbers to finally produce 8000 to 10000 seedlings at each growing period. The outcome of inoculation trials revealed that the most successful method for the establishment of ectomycorrhizal (ECM) fungi on alder roots was the addition of soil (originating from the priority habitat in Andros) in growing pots at the nursery (as opposed to the use of cultures of ECM fungi); such soil samples were found to be rich in mixed populations of symbiotic microorganisms. Therefore, alder seedlings were sowed in a mixed substrate of alder natural soil with commercial plant growth medium. As assessed -through root sampling and examination- prior to final transplanting, all alder seedlings were naturally colonized by their symbiotic actinobacteria (the genus *Frankia* was the dominant), while the majority of seedlings also hosted a variety of ECM fungi as a result of using soil inocula. About 3500 seedlings (from the first sowing phase, 1st batch) were finally transplanted and fenced at selected restoration sites in the areas of Vori and Lefka (priority habitat 91E0*). Implementation of Action C.2 will continue with inoculation and transplanting of ca. 10000 alder seedlings for completing restoration activities in the target areas.

Περίληψη

Τα αλλουβιακά δάση σκλήθρων της Άνδρου έχουν υποστεί εκτεταμένες ζημιές, οι οποίες είχαν επίσης δυσμενείς επιδράσεις στη δομή των κοινοτήτων και στους πληθυσμούς των συμβιωτικών μικροοργανισμών καθιστώντας έτσι πολύ δυσχερή τη φυσική αναγέννησή των δένδρων. Σε αυτές τις περιοχές, αποφασίστηκε η αποκατάσταση των σκλήθρων (*Alnus glutinosa*) μέσω της στοχευμένης φύτευσης εμβολιασμένων φυτών με συμβιωτικούς μικροοργανισμούς, προκειμένου να ενισχυθεί η ικανότητα τους για άμεση προσαρμογή και επιβίωση. Κατά τη διάρκεια της περιόδου 2017-2019 πραγματοποιήθηκαν πολυάριθμες εκδρομές και διάφορες δράσεις, οι οποίες -μεταξύ άλλων- αφορούσαν τη συλλογή των σπερμάτων σκλήθρου και των μυκητιακών εμβολίων, τη στρωμάτωση των σπερμάτων και την άρση του ληθάργου τους, τις δοκιμές εμβολιασμού, την ανάπτυξη των φυτών και τον τελικό εμβολιάσμο τους καθώς και τη μεταφύτευση των νεαρών δενδρυλίων στις περιοχές αποκατάστασης. Επαρκείς ποσότητες σπερμάτων σκλήθρου συλλέχθηκαν δύο συνεχείς χρονιές (φθινόπωρο του 2017 και του 2018), στρωματώθηκαν και σπάρθηκαν σε μεγάλους αριθμούς για να παραχθούν τελικά 8000 έως 10000 φυτά σε κάθε περίοδο ανάπτυξης. Τα αποτελέσματα των δοκιμών εμβολιασμού έδειξαν πως η πλέον επιτυχής μέθοδος για την εγκατάσταση εκτομυκορριζικών μυκήτων στα ριζίδια των σκλήθρων είναι η χρήση μικρών ποσοτήτων χώματος (προερχόμενου από τον οικότοπο προτεραιότητας της Άνδρου) στα δοχεία μεταφύτευσης στο φυτώριο (αντίθετα, η χρήση καλλιεργειών εκτομυκορριζικών μυκήτων δεν ήταν το ίδιο αποτελεσματική). Τα εν λόγω εδαφικά δείγματα βρέθηκαν να διαθέτουν επάρκεια μικτών πληθυσμών από συμβιωτικούς μικροοργανισμούς. Κατά συνέπεια, τα δενδρύλια των σκλήθρων μεταφυτεύτηκαν σε μίγμα αποτελούμενο από χώμα προερχόμενο από το φυσικό βιότοπο και εμπορικό υπόστρωμα ανάπτυξης. Όπως προσδιορίστηκε μετά από δειγματοληψίες ριζιδιων και εξέταση τους πριν την τελική μεταφύτευση στο πεδίο, όλα τα δενδρύλια σκλήθρων ήταν αποικισμένα από συμβιωτικά ακτινοβακτήρια (με κυρίαρχο το γένος *Frankia*), ενώ η πλειοψηφία τους είχε επίσης αποικιστεί από διάφορους εκτομυκορριζικούς μύκητες ως αποτέλεσμα της χρήσης εδαφικών εμβολίων με μικτούς πληθυσμούς. Τελικά, περί τα 3500 δενδρύλια σκλήθρου (προερχόμενα από την πρώτη φάση σποράς, 1η παρτίδα) μεταφυτεύθηκαν και περιφράχθηκαν σε επιλεγμένες θέσεις αποκατάστασης στις περιοχές Βόρης και Λεύκας (οικότοπος προτεραιότητας 91E0*). Η υλοποίηση της Δράσης C.2 πρόκειται να συνεχιστεί με τον εμβολιασμό και τη μεταφύτευση περίπου 10000 δενδρυλίων ώστε να ολοκληρωθούν οι σχετικές δραστηριότητες.

Action C.2

Deliverable: "Final Report on the alder seeds, the fungal (ECM) inocula and the alder seedlings produced for the restoration of alluvial forest in priority habitat 91E0*"

Introduction

Intense flooding phenomena are responsible for serious degradation of the *Alnus glutinosa* alluvial forests in 91E0* of Andros island during the last few years. Alder stands have suffered extensive damage, which destroyed or significantly deteriorated the structure of this unique habitat and rendered the natural regeneration of alder trees very difficult (or even almost impossible in some sites). At these particular sites, re-establishment of the population of *A. glutinosa* (and its associated ectomycorrhizal fungi) was decided to be performed through targeted planting of alder seedlings colonized with their symbiotic microorganisms to enhance their adaptability and long-term viability (Actions C.2 in conjunction with A.1 and C.3). This Action included the collection of the alder seeds and the fungal inocula, seed treatment prior to germination, growth of the young seedlings, development of microbial inocula, inoculation of seedlings and their final transplanting in the restoration areas. All previously mentioned activities were implemented during two consecutive years, i.e. 2018 (1st batch of alder seedlings) and 2019 (2nd batch of alder seedlings); a third year of pertinent activities might follow in case that additional seedlings will be needed to cover the requirements of this Action.

Methodology and implementation

Alder trees produce seed cones, each cone containing numerous seeds which grow and mature at late autumn (or earlier, depending on climatic fluctuations). For two consecutive years (2017, 2018), alder populations of Andros island were closely monitored and seeds collections were performed at the appropriate time periods. Eventually, two field-trips were performed (one each

year: 23-28/11/2017, 23-25/11/2018) for this specific purpose, and the required quantity of seeds were collected from the priority habitat 91E0* (Fig 1a,b). Collection took place in mountainous (>500 m a.s.l.; Evrousies) and coastal (0-250 m a.s.l.; Vori) *A. glutinosa* stands to obtain seeds at different state of maturity. Alder cones were manually collected from the trees at a height of ca. 2-4 m and were placed into well-aerated cloth bags.

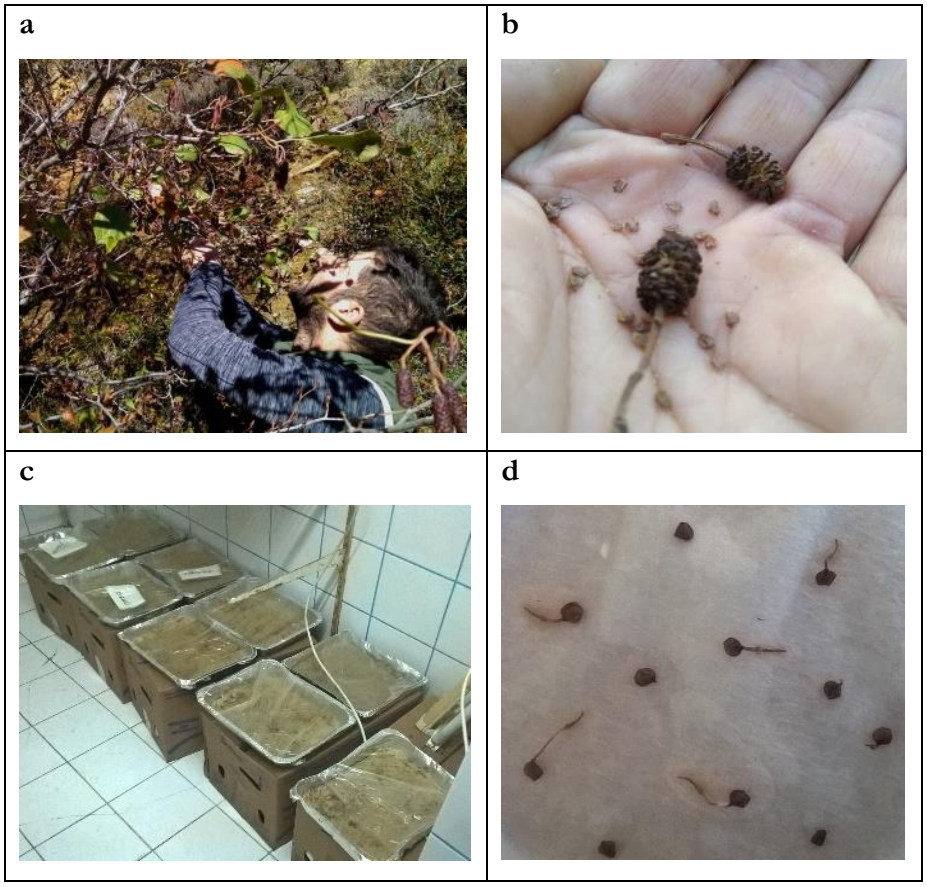

Fig. 1. a,b) Collections of alder cones, **c)** Wet stratification **d)** Germination tests.

The cones were transferred at the facilities of the Agricultural University of Athens (AUA), and after they were left to dry for a few days in the open air, seeds were extracted from the cones by mechanical means. Seeds were then placed in wet sterilized silica sand (Fig. 1c) at 4° C in the refrigerator for breaking their dormancy (wet stratification). At the same time, seeds germination tests (Fig. 1d) were conducted in Petri dishes, before, during and after wet stratification for assessing their germination index; the latter was subsequently determined to be approx. 50%. After stratification, and when the climatic conditions were favorable, the seeds were sowed in seeding

trays made of perforated thin plastic, sized 39.0 x 29.5 cm with 35 sowing compartments each. The size of the compartment is 5x5x5.5 cm and has a substrate capacity of ca. 140 ml (Fig. 2a). Seeds were left to germinate and emerging seedlings to grow for approximately three months at AUA's nursery (Fig. 2b). At that stage, the young seedlings were transported to the newly-established nursery at Agadaki Estate (please see the respective deliverable, Action C.2), where they were transplanted to pots (approx. vol. 3 lt) in order to grow and acclimatize prior to their final planting at the selected sites. Because of the mediocre ability of alder seeds to germinate (as evidenced by their germination index) and their relatively small size, 2-3 seeds were sowed in every compartment, and the most robust and healthy seedlings were kept after their emergence. In addition, each seeding tray was placed inside a plastic crate to facilitate transportation (Fig. 2c).

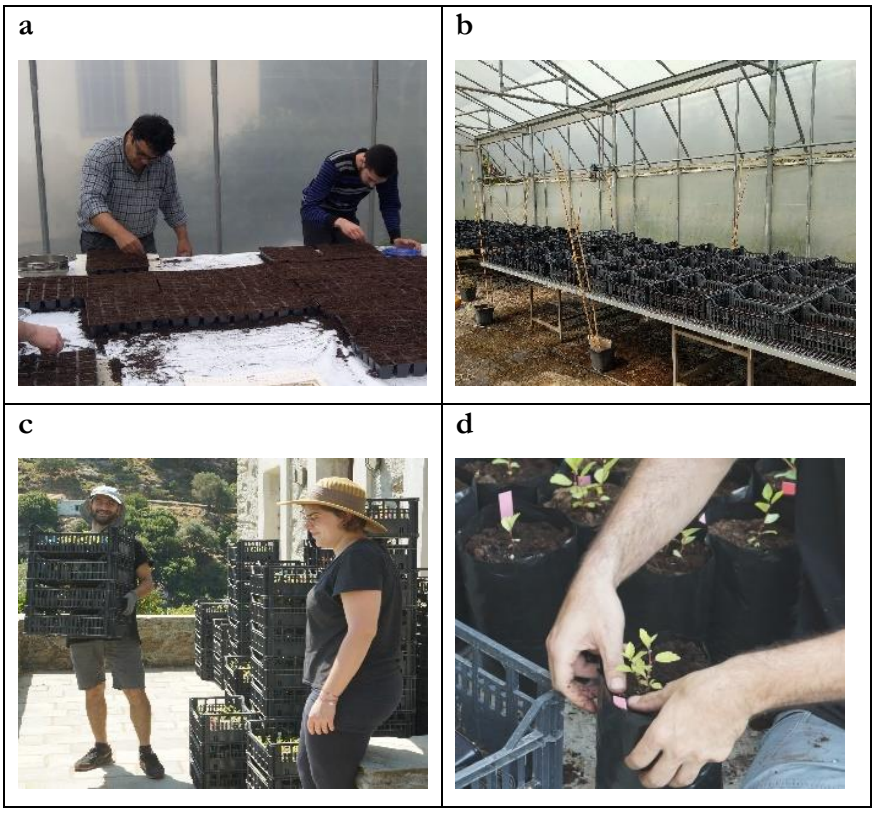

Fig. 2. a) First sowing of alder seeds, **b)** Propagation of alder seedlings at AUA facilities, **c)** Arrival of alder seedlings at Agadaki Estate, **d)** Transplanting of alder seedlings in larger pots.

For the 1st batch of seedlings produced (year 2018), the substrate commonly used for seed germination and early grow of seedlings was a commercial organic medium composed from coconut, blond and brown peat, sand and dolomite.

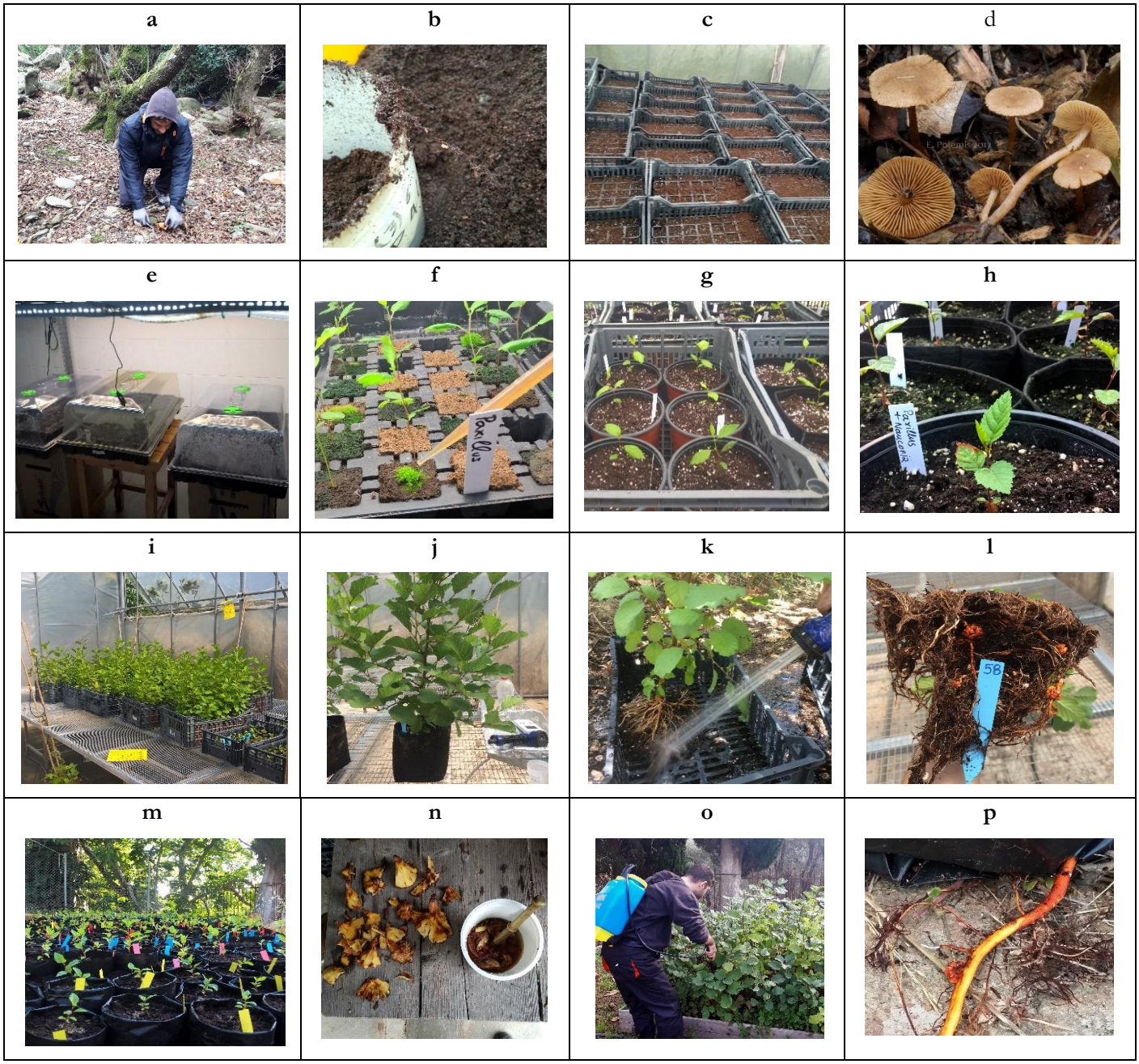

Fig. 3 a) Collection of *P. olivellus* inocula, **b)** Inoculation of alder seedlings – Mixing alder natural soil and root fragments with commercial substrate, **c)** Inoculation of alder seedlings – Snapshot of the propagating seedlings in alder natural soil. **d)** Photos of *Naucoria* sp. inocula **e)** Small scale inoculation – Growth chamber, **f)** Small scale inoculation – Inoculation with spore suspensions, **g)** Small scale inoculation – Transplanting after three months in AUA's nursery, **h)** Small scale inoculation – Growth after 3 months, **i)** Small scale inoculation – Growth after 6 months **j)** Small scale inoculation – Growth pot before colonization assessment, **k)** Small scale inoculation – Colonization assessment procedure **l)** Small scale inoculation – Alder rooting system with distinct actinobacteria nodules, **m)** Inoculation by using alder natural soil (seedlings marked with yellow ribbons) – Snapshot from Agadaki Estate nursery, **n)** Large scale inoculation – Preparing inocula by slicing hymenophores from *P. olivellus* basidiomes (ECM fungus), **o)** Large scale inoculation – Spraying seedlings with inocula, **p)** Large scale inoculation – Alder roots colonization assessment: rooting system with distinct actinobacteria nodules and ECM root tips.

However, 500 seedlings were prepared at a 1:1:1 mix of the commercial organic medium, vermiculite and natural soil obtained from the priority habitat in Andros. At the Agadaki Estate nursery, the seedlings were transplanted in larger plastic pots (Fig. 2d) containing a commercial organic substrate made of blond and brown peat moss, sphagnum moss, perlite and vermicompost mixed with extra perlite, coconut fibers, vermiculite and geolite. The young seedlings were finally transplanted and fenced at selected restoration sites in the areas of Vori and Lefka (March 2019).

Suspensions of basidiospores or hymenophore fragments were obtained from fresh basidiomes (mushrooms); their preparation process was according to the general guidelines adopted for developing inocula in commercial applications of ECM fungi (e.g. cultivation of truffles), which were suitably modified. Inocula originated from basidiomes of ECM fungi or from cultures obtained from the alder soil rhizosphere and root fragments, as described in the pertinent Deliverable (A.1; "preparation of inocula for alder seedlings"). Basidiomes were collected from the priority habitat, while environmental samples (alder rhizosphere soil and root fragments) derived from positions adjacent to *Alnus glutinosa* trees and/or from where the presence of ectomycorrhizal mushrooms was detected (Fig. 3a). Natural soil was collected from different sites within the priority habitat during the previously-mentioned sampling process. Soil and root parts were obtained after sieving which was performed during the ECM root tips examination procedure; they were then homogenized and stored (at 4°C) until further use (Fig. 3b,c).

In total, three inoculation methodologies with ECM fungi were examined: (a) small-scale inoculation of alder seedlings was performed by preparing suspensions of basidiospores and hymenophore fragments obtained from ECM fungi (*Paxillus olivellus* and/or *Naucoria* spp.) and by applying them at the early growth stages of alder seedlings placed into growth chambers at AUA's premises (Fig. 3d,e), while large-scale inoculation was implemented either (b) through the use of *P. olivellus* hymenophores suspension applied at the soil surface of pots with plants growing at the Agadaki Estate (after their first transplanting), or (c) by using natural soil from the priority habitat and applying it to small pots or trays where alder seeds were sowed (at AUA's nursery; 500 seedlings of the 1st batch and all seedlings of the 2nd batch).

Especially as regards small-scale inoculations, in the case of mushrooms of the genus *Naucoria*, suspensions derived from entire basidiomes due to their small size, whereas fragments of hymenophores were sufficient for preparing suspensions of *P. olivellus*. Suspensions (4 ml each) were applied three times during *A. glutinosa* early growth stages (Fig. 3f) either immediately after their preparation or after being stored (4°C) for a short period. During trials, plants were inoculated

either by *P. olivellus* or by *Naucoria* spp. suspensions, or by a suspension containing both of them; part of seedlings were not inoculated and were evaluated as control. After a three-month growth period at controlled conditions (inside the growth chamber), the seedlings were transferred in AUA's nursery, where they were transplanted into larger pots (Fig. 3g,h). Seedlings were left to grow for extra three months, and then examined for colonization by ECM fungi (Fig. 3i-l). As regards large-scale inoculations, hymenophore suspensions deriving from *P. olivellus* basidiomes were applied by spraying directly onto the soil surface of pots with plants maintained at Agadaki Estate (Fig. 3n,o). Approx. three months after inoculation, a representative sample of seedling was assessed for ECM root colonization (Fig. 3p).

As regards the 2nd batch of seedlings produced (year 2019), all plants were produced at a 1:1 mix of the commercial organic medium and natural soil obtained from the priority habitat in Andros. Seeds sowing was performed at early March of 2019 and their growth is in progress at AUA's nursery.

On the basis of the alder seeds average weight, it is estimated that a few hundred thousand seeds were collected and stored during the first two years. Approx. 40000 seeds were sowed at the first year (four-five seeds per compartment in ca. 8500 sowing compartments) and approx. 20000 seeds were sowed at the second year (two-three seeds per sowing compartment). As regards the first batch of seedlings produced (2018), ca. 9000 seedlings were transferred to Andros in early June to be transplanted in larger pots, but due to the adverse weather conditions that prevailed at the end of June (very high temperatures and hail storms), only 3500 of them finally survived. These seedlings were inoculated as previously described, and transplanted at the selected restoration sites in early March 2019. The final transplanting was performed along the shores of the Vori (500-1000 seedlings) and Lefka (ca. 2500 seedlings) streams, and it was accompanied by wire fencing to protect the young plants from grazing by feral goats (activities implemented by the AUA and MA teams) (Fig. 4a,b,c,d).

Inoculated trees were examined after three (large-scale inoculation) or six months (small-scale inoculation) to ascertain their colonization state. The most successful method for ECM establishment was adding soil from the priority habitat, which resulted in alder roots colonization with several different ECM morphotypes thus exhibiting far better results than by inoculation through the use of spore/basidiome/hymenophore suspensions (in the latter case, only traces of some ECM morphotypes were observed).

Figure 4 a,b,c,d. Transplanting and fencing of the seedlings during the first restoration phase.

Hence soil amendments yielded the highest number of morphotypes, with satisfactory abundance and density of colonization. Four different ECM morphotypes were detected, i.e. two dominant which were particularly abundant and were identified as *Tomentella* sp. (Fig. 5a, b), one single root-tip with paxilloid morphology (Fig. 5c) and a (still) unidentified ECM morphotype (Fig. 5d). It is noteworthy that during large-scale inoculations by *P. olivellus* (performed on 3500 seedlings at the Agadaki Estate), two tomentelloid ECM morphotypes were detected on the majority of the examined plants and were mainly localized at seedlings external rooting system (Fig. 3p). These morphotypes were the same as those detected when alder soil was used for inoculation purposes. It is quite possible that seedlings were colonized by other locally occurring ECM fungi (probably with a broad host-range spectrum, e.g. *Tomentella* spp.) during their stay at the Agadaki Estate

nursery, which may have prevented the colonization of roots by *P. olivellus* inocula used in large-scale trials.

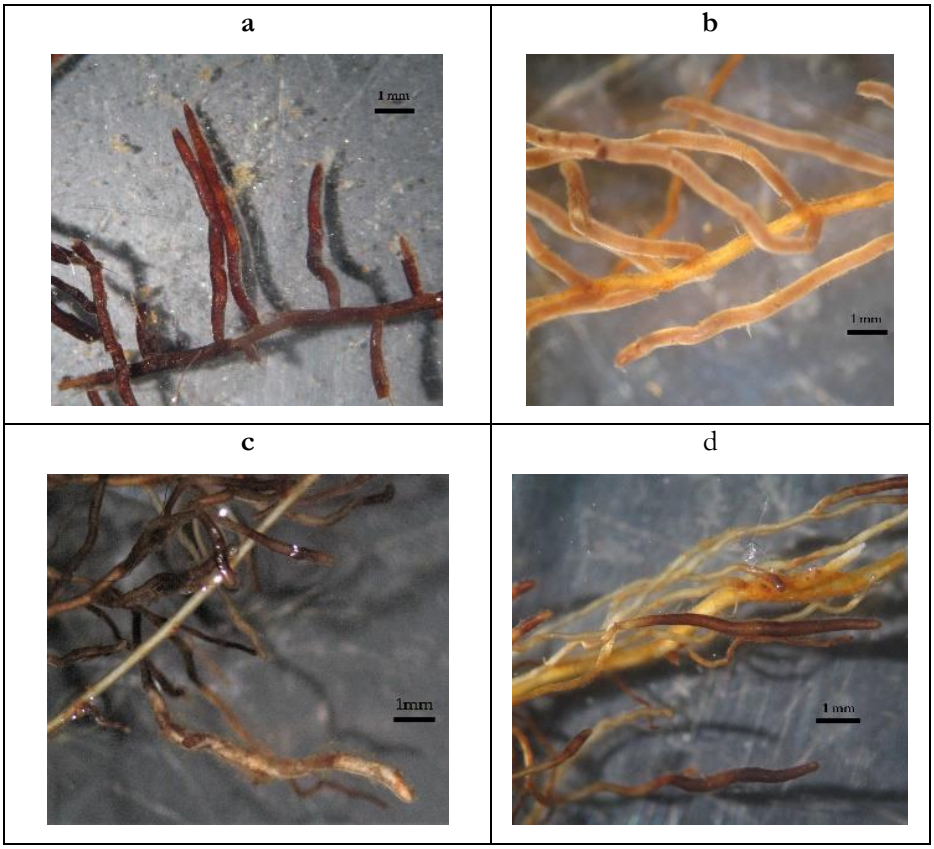

Fig. 5 a, b) Dominant morphotypes identified as *Tomentella* sp. **Fig. 5c)** One single paxilloid morphotype among dominant tomentelloid morphotypes **Fig. 5d)** Non-identified ECM-root morphotype.

Moreover, actinobacteria nodules responsible for nitrogen fixation were also abundant on every alder root examined irrespectively of whether the trees were inoculated or not (Fig 3l). The non-inoculated control seedlings growing in pasteurized commercial soil also presented a high number of actinobacteria nodules, indicating that these symbiotic microorganisms exist as endophytes and are present in the seeds (and subsequently transferred to the growing plant).

From all above-mentioned data, it is obvious that seeds sowing and seedlings growth in alder natural soil is the fastest and safest way to accomplish alder colonization with a variety of microorganisms, which would in turn facilitate their subsequent establishment and survival at the restoration sites. Therefore, it was decided to use this particular approach for the 2nd batch of seedlings, and the pertinent process is in progress.

Important Dates

1st sowing (1st batch) of alder seedlings (medium-scale inoculation of 500 seedlings during test trials)

- 22-26/11/2017: Collection of alder cones from two sites (Evrousies and Vori).
- Cones drying and extraction of seeds at AUA's facilities.
- 18/1/2018: Wet stratification of seeds in sterilized silica sand at 4 ^{0}C.
- Seeds germination tests, before, during and after wet stratification for assessing their germination index.
- 9/3/2018: Sowing alder stratified seeds in sowing trays of approx. 9000 sowing compartments.
- Developing of extra 500 seedlings in a substrate mix (1:1:1) consisting of commercial medium, alder natural soil and vermiculite.

2nd sowing (2nd batch) of alder seedlings (large scale inoculation of the new seedlings)

- 23-25/11/2018: Collection of alder cones from two sites, Evrousies and Vori.
- Cones drying and extraction of seeds at AUA's facilities.
- 6/2/2019: Wet stratification of seeds in sterilized silica sand at 4 ^{0}C.
- 27-28/3/2019: Sowing alder stratified seeds in sowing trays and development of approx. 10000 seedlings.
- Seeds were sowed in a substrate mix (1:1) consisting of commercial medium and alder natural soil.

Inoculation of alder seedlings

Small-scale inoculation

- 22/1/2018: Sowing approx. 80 seeds in hydroponic sponges inside growth chambers.
- 22-24/2/2018: Collection of *P. olivellus* and *Naucoria* spp. mushrooms from Vori.
- 26/02/18: Preparing inocula suspensions (4 ml) from hymenophores, entire basidiomes and/or spores of ECM fungi, and application to seedlings
- 16/3/18: Inocula suspensions applied to seedlings
- 28/3/2019: Inocula suspensions applied to seedlings

Large-scale inoculation

- 23-25/11/2018: Collection of *P. olivellus* mushrooms to prepare suspensions of hymenophore fragments to inoculate 3500 seedlings at Agadaki Estate.
- 27-28/3/2019: Generalized use of soil (deriving from the priority area) as inoculum for ca. 10000 seedlings growing in AUA's premises.

Conclusions

Alder seeds collection, development of alder seedlings and root colonization by symbiotic fungi was performed as anticipated. Alder trees were rather easily propagated and grown under nursery conditions, while establishment of symbiosis with ectomycorrhizal (ECM) fungi is fast if they occur at the same biotope or if care is taken to inoculate with the suitable biological material. Although initially one of the main targets was to establish ECM symbiosis with young alder seedlings by isolating autochthonous fungi in pure cultures and then using them as inoculants, the difficulty/delay at making them produce mycelium and grow at satisfactory rates (which would permit colonization of the alder roots in the timeframe set by the project) rendered this approach not suitable for our purpose. Instead, an alternative approach was adopted, i.e. using of soil (containing ECM inoculants) obtained from the rhizosphere of alder trees to mass inoculate young seedlings at the nursery. This proved to be the best (fastest and safest) way for implementing large-scale inoculation which would consequently ascertain forest restoration. It is worth mentioning that natural soil hosts a large range of alder symbiotic microorganisms, including plant growth promoting fungi or bacteria, which facilitate seedlings growth at a considerably larger extent than by using just one microorganism through controlled inoculation. In conclusion, the alder seedlings used in the restoration Action C.2 were colonized by several different symbiotic microorganisms before their final transplanting in the field, thus their adaptivity was enhanced as initially envisaged. The success of this approach was verified through the inspection of an adequate number of alder seedlings; in all cases, the presence of several ECM morphotypes was detected on the roots.