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Effect of mycorrhiza application on vitality of *in vitro* propagated *Prunus avium* clones during acclimatization

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ABSTRACT

The effect of mycorrhiza application on vitality – i.e. survival and growth – has been investigated on *in vitro* propagated *Prunus avium* clones. In two experiments altogether 36 clones of *P. avium* were treated with arbuscular mycorrhiza and compared with uninoculated control plants. Inoculation was carried out during and after acclimatization when plants were potted for further greenhouse cultivation, respectively. Survival of the plants inoculated during acclimatization was surveyed after three months. Height and survival of potted trees were assessed at the end of the growing season. 53 % of tested clones showed a positive effect on vitality with an increase of the survival rate up to 70 %. Height was increased up to threefold in 77 % of the tested clones. A low degree of root colonization seems to increase survival of mycorrhized plants.

INTRODUCTION

The increasing need for high value timber is a big challenge for future forestry and will lead to agroforestry plantations of fast-growing elite trees. Increased harvest, quality and health are very important breeding aspects for the domestication of forest trees but difficult to achieve due to the long reproduction cycles (Fladung, 2008). Selection and vegetative propagation of elite clones has already led to production of clonal wild cherry (*Prunus avium*), hybrid birch (*Betula pendula* x *B.*

platyphylla var. *japonica*) and shipmast Robinia (*Robinia pseudoacacia* var. *rectissima*). Some of these selected trees are produced under the German trademark "silvaSELECT®" (Meier-Dinkel, 2007). Owing to the high economic value the interest in growing clonal trees is increasing especially for *P. avium*. The method for *in vitro* propagation of *P. avium* was established by the former Lower Saxony Forest Research Institute already in 1984 (Meier-Dinkel, 1986). Today 26 clones are certified and registered for propagation. However, mass production still needs to be optimized. Especially during acclimatization the loss of plants is comparatively high. Recent results show that the mean production success (ready-to-be-sold trees/number of *in vitro* produced microcuttings) is less than 30 %, whereas with Robinia and Birch a production success of 80 and 85 %, respectively, can be achieved, (Gruß, 2008). 50 % of the registered *P. avium* clones show a production success below 30 %. According to the German Law on Forest Reproductive Material a clonal mixture has to be delivered with approximately the same portion of plants per clone. For an economically successful production of all clones survival during production must be increased.

Arbuscular mycorrhiza fungi (AMF) have shown positive effects on the acclimatization of *in vitro* propagated tree species (Berta et al., 1995; Fortuna et al., 1996; Moraes et al., 2003; Binet et al., 2007) and especially for *P. avium* (Cordier et al., 1996; Grange et al., 1997). In our experiment the application of arbuscular mycorrhiza fungi was tested on altogether 36 clones. We wanted to find out if AMF would have a general effect on our *in vitro* propagated *P. avium* clones concerning plant growth and survival, where a positive effect would help saving costs in the production process of high value timber trees.

MATERIAL AND METHODS

In vitro cultivation of *Prunus avium*

Propagation of clones was carried out according to Meier-Dinkel (1986) in glas jars (250 ml, Weck) on a MS nutrient medium (Murashige & Skoog, 1962) with 0.5 mg/l Benzylaminopurine (BAP), 0.1 mg/l Indole-3-butyric acid (IBA) and 0.1 mg/l Gibberellic acid (GA_3). The pH was adjusted to 5.8. Subcultures were carried out every four weeks. Cultures were kept in a climate chamber at 24 ± 1 °C and a ratio of light : darkness of 16 h : 8 h. Light intensity was 1200 lux.

For elongation of the shoots 20 ml of hormone-free liquid medium was added to the cultures two weeks after subcultivation. Two weeks after this treatment, microcuttings were harvested and transferred to rooting medium (MS at 1/3 strength with 1.0 mg/l IBA). Two weeks later, microcuttings were transferred to the greenhouse.

Production of mycorrhizal inoculum

As arbuscular mycorrhizal inoculum INOQ Agri (**www.inoq.de**) was chosen. The inoculum was produced according to the directed inoculum production process (DIPP, Feldmann & Grotkass, 2002) on vermiculite as carrier. Quality control was carried out according to the agreement of the

Committee of Mycorrhiza Application in Germany (von Alten et al., 2002). The mycorrhiza inoculum showed the following characteristics:

Table 1 Characteristics of the arbuscular mycorrhiza inoculum for application to *in vitro* propagated *Prunus avium*

Test Parameter	Value
Mycorrhizal fungi, native strains, do not contain genetically modified organisms	*Glomus etunicatum* *G. intraradices* *G. claroideum*
Most probable number of propagules (MPN) [n/cm^3]	205 ± 19
Effectiveness (MEI)	**26 ± 8**
Carrier material and grain size [mm]	Vermiculite, 1 - 2
specific weight [g/l]	530 - 560
pH	5.7
Content of fertilizer of substrate [mg/100 g DW]	
Nitrate-Nitrogene, Ammonium-Nitrogene	7; 0.5
Phosphate (P_2O_5), Potassium (K_2O)**,** Magnesium (Mg)	7; 147; 59
Germination inhibition	**none**
Fungal contaminants *(Athella rolfsii, Botrytis cinerea, Colletotrichum spp. (C. coccodes, C. acutatum), Didymella spp., Fusarium spp. (F. solani, F. oxysporum), Penicillium spp., Phoma destructiva, Phytophthora spp. (P. capsici, P. cinnamomi, P. drechsleri, P. cryptogea, P. infestans, P. nicotianae, P. ramorum, P. fragariae, P. cactorum), Plectosphaerella cucumerina, Pyrenochaeta lycopersici, Pythium spp. (P. aphanidermatum, P. dissotocum, P. polymastum, P. sylvaticum, P. ultimum, P. irregulare), Rhizoctonia solani, Sclerotinia spp. (S. minor, S. sclerotiorum, S. trifoliorum) Cylindrocladium spp., Thielaviopsis basicola, Trichoderma spp. (T. asperellum, T. harzianum, T. hamatum), Verticillium spp. (V. albo-atrum, V. dahliae)*	none
Bacterial contaminants *(Agrobacterium tumefaciens, Pseudomonas spp. (P. marginalis, P. cichorii, P. viridiflava, P. syringae, P. syringae pv. Porri), Xanthomonas fragariae, Ralstonia solanacearum)*	none
Potential phytophageous faunistic contaminants	
Diptera, Coleoptera, -larva, Collembola, Acari, Nematoda, Gastropoda	**none**
Botanical contaminants	
Algae (Diatomeae, Cyanophyceae, Chlorophyceae)	**present**
„Weeds“	**none**
Tolerance of fungicides	**proven**

Methods: pH and content of fertilizer analyzed by LUFA, Hameln, Germany;
MPN and Bioassays carried out by Institut für Pflanzenkultur, Solkau, Germany
Fungal and bacterial contaminants analyzed by DNA multiscan®, Germany/Belgium

Inoculation during acclimatization

Micropropagated plantlets of 18 clones were planted into 4 x 4 cm Jiffy Strips in a substrate mixture of conventional peat compost, perlite and 5 % v/v INOQ Agri. They were kept in a greenhouse under a plastic tent with high humidity (> 90 %) and a temperature of 20 to 24 °C. Humidity was decreased after four weeks by partially lifting and later removing the plastic tent. Survival rates were investigated after three months.

Inoculation after acclimatization for further greenhouse cultivation

Micropropagated plantlets of another series of 18 clones were acclimatized in the greenhouse as described before, but without mycorrhiza inoculation. After three months the acclimatized plants

were potted into 1,3 l Kitty Plast containers in conventional peat substrate and inoculated with 30 ml of INOQ Agri. Vitality and height were assessed at the end of the growing season, i.e. after four months. The Degree of Root Colonization was investigated in eight clones.

Root investigation

Roots of the plants were investigated after the modified protocol of Phillips & Hayman (1970):

Fixation:	Ethanol : Acetic acid (6 : 1).
Clearing:	1 hour in 10 % (w/v) KOH at room temperature
Washing:	0.1 N HCl
Staining:	0.05 % (w/v) Trypaneblue in waterfree Glycerine : Lactic Acid, 1 : 1
Storage:	Glycerine : Lactic Acid , 1 : 1

The investigation of the Degree of Root Colonization was carried out after Trouvelot (1986) and measured as Frequency of Root Colonization (%F).

RESULTS

Effect of mycorrhization during acclimatization

The investigation of the effect of the mycorrhization on survival rates during acclimatization showed the following results: There was a positive effect of mycorrhiza treatment on eight clones, and a negative effect on ten clones. Only clone 58-7 of the silvaSELECT®-clones showed a positive effect after application of AMF whereas the clones 58-21, 58-18, 58-3, 134-9 and 134-33 of the selected clones showed negative effects of AMF (Fig. 1).
The mean survival rate of control and treated plants showed a slightly positive effect of AMF (Fig. 1).

For further investigation of the effect of mycorrhization the difference of the survival rate between inoculated and control plants was calculated. In this experiment, AMF only had a positive effect on weaker clones with low survival rates of the controls. The higher the vitality of the clones was the lesser the effect of AMF turned out.

The evaluation of the effect of mycorrhization between inoculated and control plants showed that plants of three clones with survival rates below 60 % in the control showed a statistically significant increase of survival after AMF treatment from 24 to 38 %. As soon as survival rates of untreated plants were higher than 80 % only negative response to AMF treatment was observed. Untreated clones with 100 % survival rate had the highest negative effects of AMF (Fig. 2).

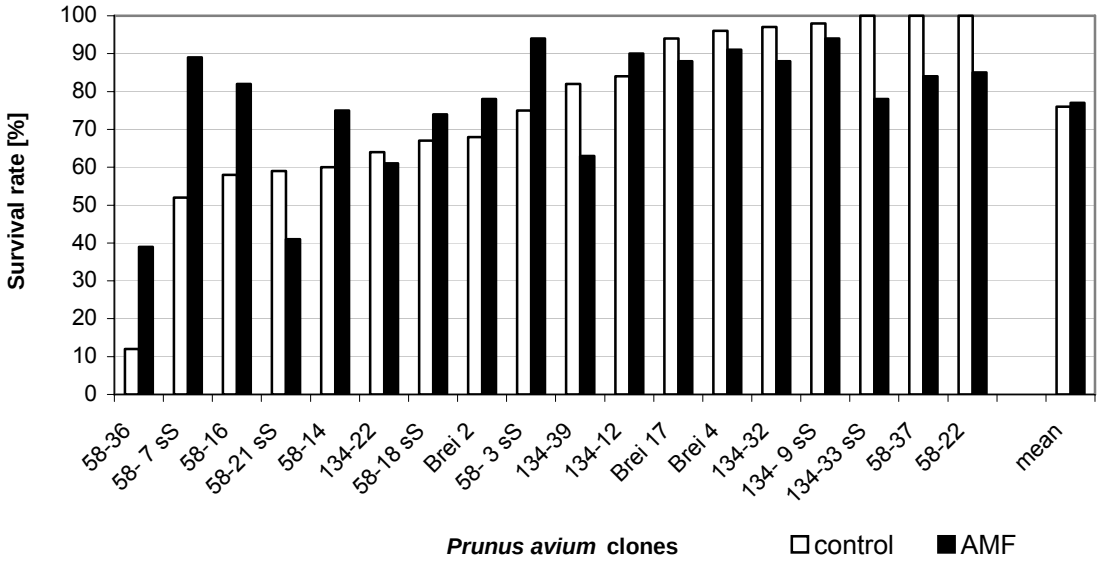

Figure. 1: Survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants, n = min. 32 (to 100) Inoculation was carried out during acclimatization, measurement of survival rate three months later
sS= silvaSELECT-clones

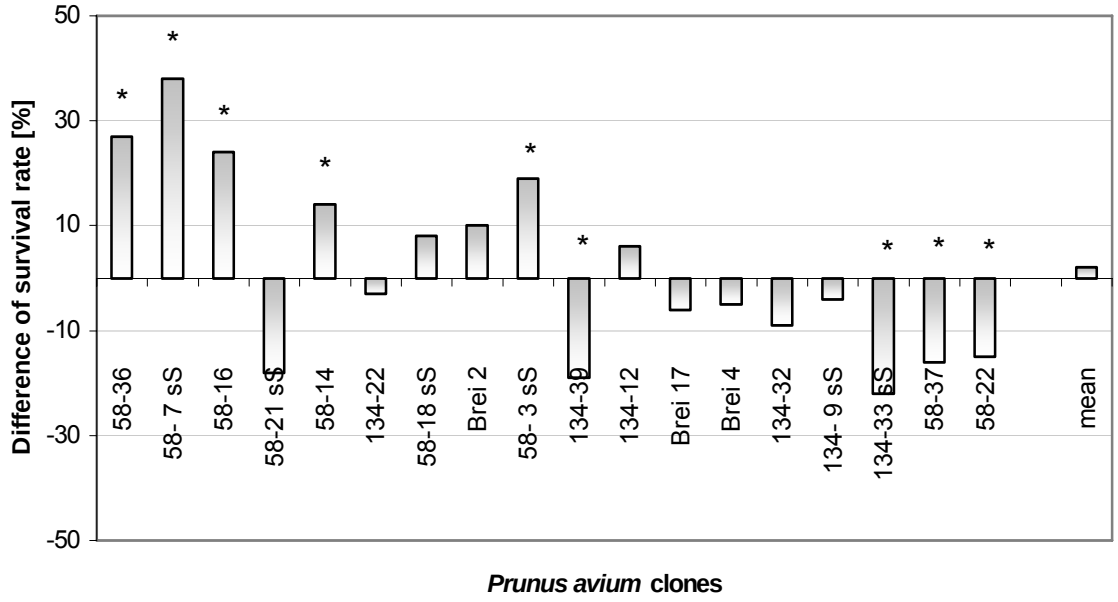

Figure. 2: Difference of survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants, n = min. 32 (to 100), Inoculation was carried out during acclimatization, measurement of survival rate three months later, * = statistically significant after Fisher's exact test for the analysis of variance in a 2*2 contingency table, sS= silvaSELECT-clones

Effect of mycorrhization after acclimatization

The investigation of the effect of mycorrhization on the survival rates of *P. avium* after acclimatization showed similar effects: There was a positive effect on ten clones, no effect on three clones and a negative effect on five clones (Fig. 3). On three clones the survival rate was increased to 100 % after inoculation (134-32, 58-9, 134-8).

Again the effect of mycorrhization was positive on weaker clones with 60 % to 70 % survival rates of untreated control. The silvaSELECT®-clones 134-7, 134-1 and 58-15 reacted positive to mycorrhization whereas 134-6 and 134-9 showed a negative and 58-20, 58-27, and 58-1 no effect

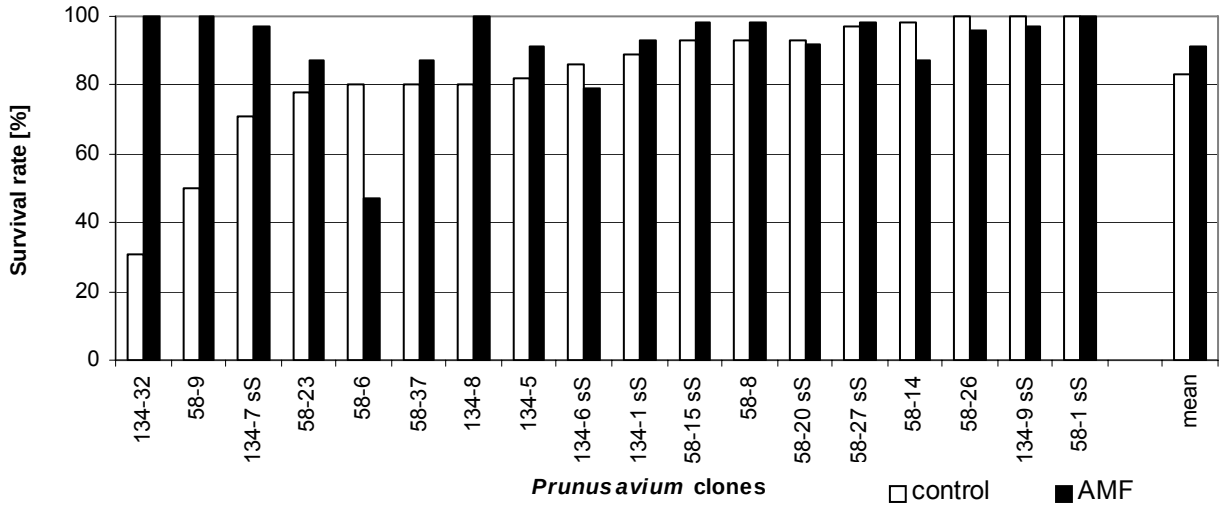

Figure. 3: Survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants, n = min. 15 (to 100)
Inoculation was carried out after acclimatization, measurement of survival rate four months later
sS= silvaSELECT-clones

For better understanding of the effect of mycorrhization the difference of the survival rates is shown in Figure 4. The increase in survival between 26 and 69 % of the three weak clones with high losses of the controls after potting is statistically significant. Only one clone, 58-6, shows a significantly negative difference of the AMF treated plants to the controls.

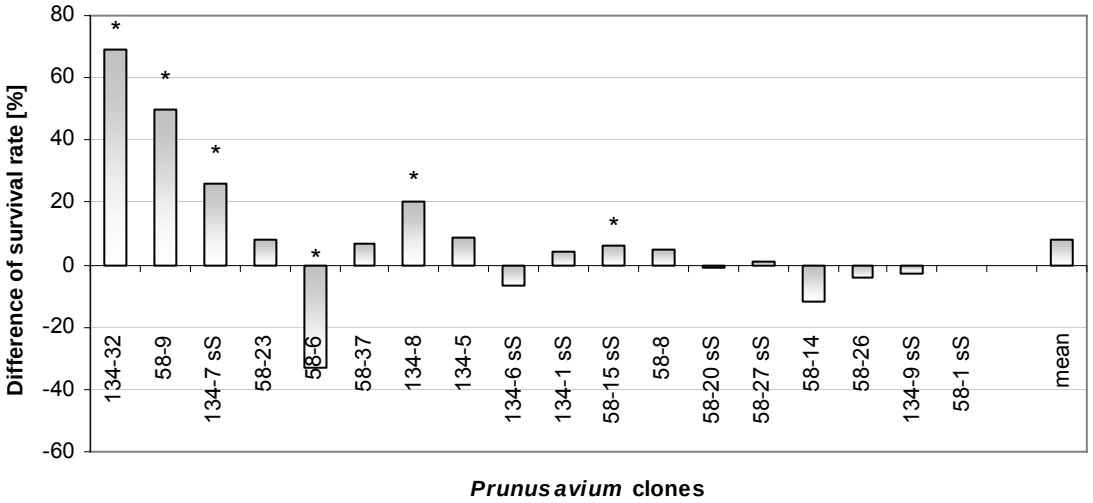

Figure. 4: Difference of survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants
n = min. 15 (to 100), Inoculation was carried out after acclimatization, measurement of survival rate 3 months later, * = statistically significant after Fisher's exact test for the analysis of variance in a 2*2 contingency table, sS= silvaSELECT-clones

On the 18 clones which were inoculated after acclimatization the height of the plants was measured after four months at the end of the growing season. 14 clones showed an increased growth after AMF treatment. One clone showed no effect and three clones had a decreased growth after inoculation. Of the silvaSELECT®-clones 134-6, 58-20 and 58-27 had a positive growth response. 134-7 and 134-9 showed a negative effect of mycorrhization (Fig. 5).

The AMF treatment lead to growth responses from – 20 % (clone 134-7) to + 262 % (clone 134-6). The mean height of the plants could be increased by 22 % through AMF treatment.

The investigation of the ratio of growth response (measurement of height) to survival rates of the mycorrhized clones can help to answer the question, if clones with f.e. increased vitality show a comparable height increase.

All clones with an increased height after AMF treatment had shown a wide range of survival rates during acclimatization. Thus survival rates of different clones do not have a predictable impact on later growth of the plants (Fig. 6).

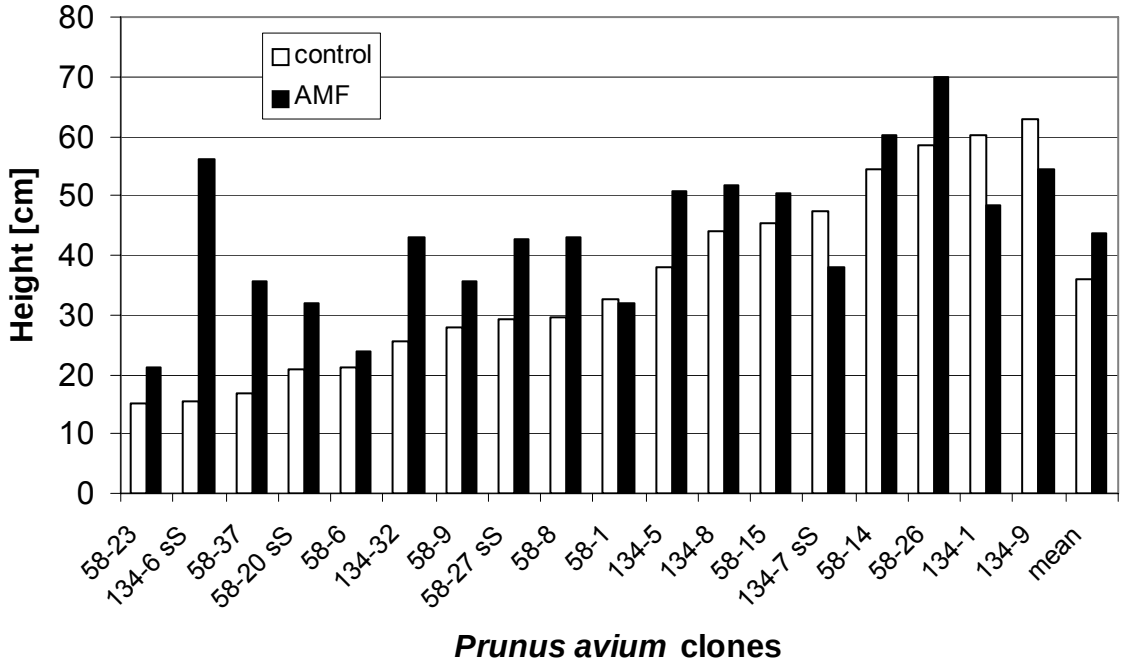

Figure 5 Effect of Arbuscular Mycorrhiza Fungi (AMF) on growth of micropropagated *Prunus avium* clones, n = min. 8, sS= silvaSELECT-clones

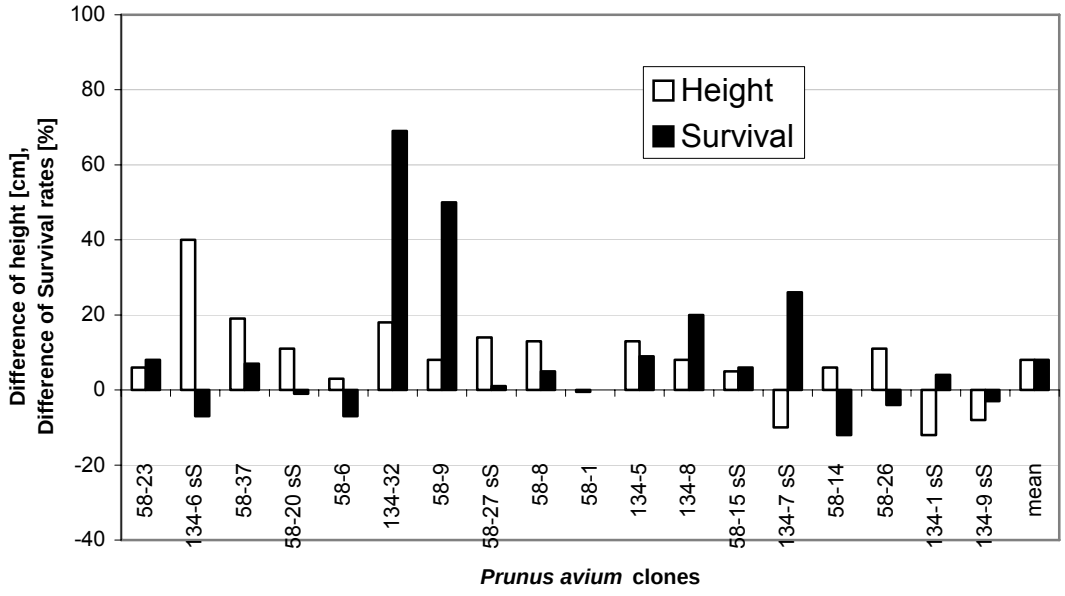

Figure. 6: Correlation of the effect of Arbuscular Mycorrhiza Fungi on growth and survival of micropropagated *Prunus avium* clones, sS= silvaSELECT-clones

The degree of root colonization (DRC, %F) was investigated in 8 clones. It ranged from 13 to 46 % with a mean of 30 %. Positive effects on survival occured with DRCs of 13 to 34 %, whereas negative effects occured with DRCs of 27 to 46 %. (Fig. 7).

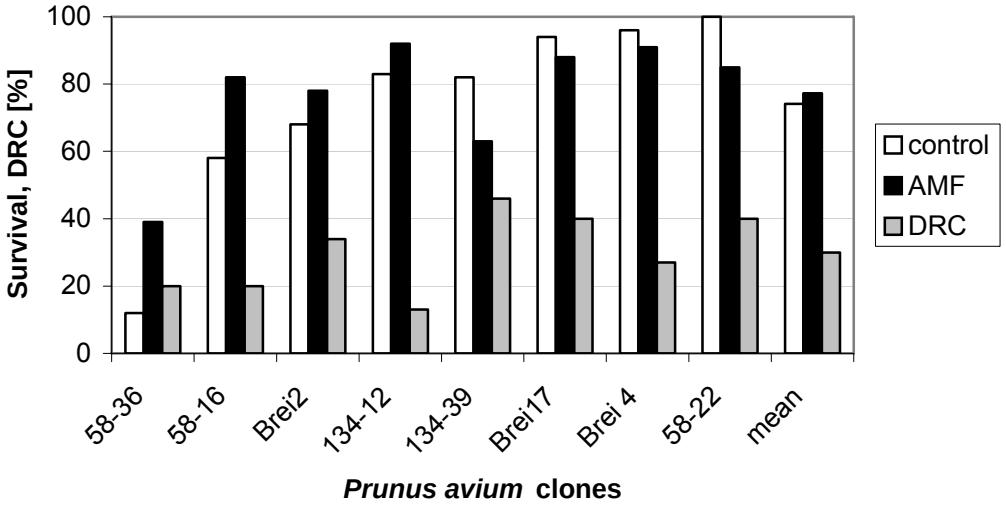

Figure 7 Degree of root colonization (DRC) of micropropagated *Prunus avium* clones in comparison to survival rate (inoculated =AMF, non-inoculated = control)

DISCUSSION

Our experiments show that AMF inoculation had positive effects on *Prunus avium* clones which showed low survival rates during and after acclimatization. Clones with high survival rates show no and negative effects, respectively. There seems to be a threshold for mycorrhizal effects, which means, that weak clones benefit more from mycorrhization. The effect of AMF on growth of tested clones was measured from -15 to +40 %. This differs to the results of Cordier et al. (1996) who found only positive AMF effects on plant growth.

Comparison of growth with survival of the tested clones showed no correlation. Clones with the highest increase of growth also had the highest increase of survival rates, but plants of clones that had shown a decrease of survival after AMF treatment also reacted with positive growth development.

Our results concerning Degree of Root Colonization show, that it cannot be generally found what degree of DRC leads to positive effects. As *Prunus avium* is very sensitive it could be, that a high DRC could weaken the plants directly after transplant stress. This again differs to Cordier's et al. (1996) results where DRCs of over 90 % occured in all variants. But repeated results in applied experiments in plant production show the same findings where positive effects of AMF treatment

on plant growth are not necessarily correlated to a high DRC. An explanation could be that due to extraradical mycelium the mycorrhized soil has a positive influence on plant development (Augé, 2003).

Cordier et al. (1996) also stated that AMF had a positive effect on growth depending on the time of inoculation. They inoculated the plants directly when transferred from laboratory to the greenhouse. In our experiment plants were inoculated directly after transfer to the greenhouse and also after acclimatization during potting. As the survival rate during weaning from *in vitro* to *ex vitro* was only 51 %, it should be considered to always inoculate at the early stage. Thus our method for inoculation of *P. avium* clones should be optimized especially because all silvaSELECT®-clones need to be propagated economically successful to fulfill the German Law on Forest Reproductive Material.

The potential for an optimization of the method is enormous: With the growing interest of the forestry in selected wild cherry clones the costs for production need to be optimized. The insufficient production success of only 30 % (ready-to-be-sold trees/number of *in vitro* cuttings) leads to a calculatory price of approximately 5,00 € per plant (plants in 2 l pots with heights up to 1,20 m), which is not tradeable in the market. With an increase of the production success to a mean maximum of 80 % this prize could be reduced to 2,00 to 2,30 €, which is a marketable value.

There is also potential to reduce costs for inoculation of plants. In our experiment 30 ml of inoculum was used for 1,3 l pots (= 0,11 €; price 2008: 3,50 €/litre inoculum). If plants were inoculated already at the beginning of the acclimatization with 5 % v/v only 3 ml of inoculum would be necessary for 60 ml pot volume. Thus the costs would be reduced to 0,01 € per plant. This calculation shows that a successful mycorrhization of plants would help saving costs in this difficult production process of high value timber trees.

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