DISTINCT AND VISIBLE DIFFERENCES IN MICROPROPAGATION OF *VACCINIUM MYRTILLUS* ESTABLISHED FROM MATURE AND JUVENILE TISSUE

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The *in vitro* propagation of *Vaccinium myrtillus* from nine Austrian sites via mature (adult) and juvenile plant material was tested in a modified woody plant medium (WPM). The *in vitro* establishment of adult shrubs via nodes (repeated over three vegetation periods) led to induction of axillary and adventive micro-shoots on plants from three accessions (Sailer (S/3), Planner/unten (PU/1) and Planneralm (PA/1b/1, PA/1c/1)) after preconditioning in pots for 12 months. Seeds from ripe berries collected at three sites (Kronsegg, Mölbegg and Raumberg1) cultivated immediately after harvest (WPM plus 3 g/l activated charcoal) germinated within 10 days of *in vitro* establishment. Germination did not occur, however, with the 8.5 month old seeds from the Kronsegg provenance. During in vitro proliferation in 1 mg/l IAA, 1 mg/l GA_3 and 2 mg/l Zeatin, micro-shoots were immediately induced on the generatively propagated seedlings, whereas on the established clones (via nodes), induction required up to 8 months. However, the latter did not show any signs of rejuvenation during the 6 to 8 week subculturing period. On the node-generated clone Sailer a maximum mean of 2.37 ± 0.27 micro-shoots/explant could be induced, in contrast to the seedling clone KR/S3 from the Kronsegg site, which achieved a maximum result of 5.81 ± 0.44. For the *in vitro* rooting of clones established from nodes (1 mg/l IBA, 1 mg/l IBA + 0.8 g/l activated charcoal, 1 mg/l NAA) there were observed time slots in which distinctly higher rooting frequencies on S/3, PU/1, PA/1b/1 and PA/1c/1 were achieved. This may be due to endogenous physiological factors. Successful rooting occurred with 2 to 4 months old cuttings from acclimatized plants while applying a 1 g/l IBA pulse treatment. S/3 showed 67 % and 100 %, PA/1b/1 100 % and 84 % rooting frequencies. Plantlets from the node-generated clones Sailer, Planner/unten, Planneralm (PA/1b/1) and from 19 seedling clones (Kronsegg, Mölbegg, Raumberg1) could be successfully acclimatized for field trials.
Keywords: In vitro propagation, Vaccinium myrtillus, nodes, seeds

Deutlich sichtbare Unterschiede bei der Mikrovermehrung von Vaccinium myrtillus aus adultem und juvenilem Gewebe. Auf einem modifizierten Woody plant-Nährboden (WPM) wurde die *In vitro*-Vermehrung von Vaccinium myrtillus von neun österreichischen Standorten über adultes und juveniles Pflanzenmaterial untersucht. Bei der *In vitro*-Etablierung adulter Sträucher über Nodien (in Wiederholungen über drei Vegetationsperioden) kam es bei den Herkünften Sailer (S/3), Planner/unten (PU/1) und Planneralm (PA/1b/1, PA/1c/1) nach einer Präkonditionierung in 12-monatiger Topfkultur zur Induktion von Axillar- und Adventivsprossen. Bei der *In vitro*-Etablierung über Samen (WPM plus 3 g/l Aktivkohle) zeigten sofort kultivierte Samen aus reif geernteten Beeren der Standorte Kronsegg, Mölbegg und Raumberg1 bereits innerhalb von 10 Tagen eine Keimung; 8,5 Monate alte Samen der Herkunft Kronsegg keimten hingegen nicht. Bei der *In vitro*-Vermehrung auf 1 mg/l IAA, 1 mg/l GA_3 und 2 mg/l Zeatin kam es bei den generativ vermehrten Sämlingen sofort, bei den vegetativ etablierten Klonen über Nodien erst innerhalb von acht Monaten zur Induktion von Mikrosprossen. Während der sechs- bis achtwöchigen Subkultur zeigten die vegetativ etablierten Klone keine Verjüngung. Beim vegetativ etablierten Klon Sailer konnte ein maximaler Mittelwert von 2,37 ± 0,27 Mikrosprossen/Explantat induziert werden. Im Vergleich zeigte der Sämlings-Klon KR/S3 des Standortes Kronsegg einen Maximalwert von 5,81 ± 0,44. Bei der In vitro- Bewurzelung (1 mg/l IBA; 1 mg/l IBA + 0,8 g/l Aktivkohle; 1 mg/l NAA) zeigten sich bei S/3, PU/1, PA/1b/1 und PA/1c/1 klar sichtbare Zeitfenster, die auf endogene, physiologische Faktoren hinweisen. Erfolgreich verlief die Bewurzelung von zwei bis vier Monate alten Stecklingen von akklimatisierten Pflänzchen nach 1 g/l IBA-Pulsbehandlung. S/3 zeigte 67 % und 100 %, PA/1b/1 100 % und 84 % Wurzelbildung. Jungpflanzen von den aus Nodien gewonnenen Klonen Sailer, Planner/unten, Planneralm (PA/1b/1) und von 19 Sämlingsklonen (Kronsegg, Mölbegg, Raumberg1) konnten erfolgreich für Feldversuche akklimatisiert werden.

Schlagwörter: *In vitro*-Vermehrung, *Vaccinium myrtillus*, Nodien, Samen

Vaccinium species play an important role in human health, especially as a dietary fruit to prevent atherosclerosis, breast cancer and osteoporosis (Wood, 2011). Beneficial anti-inflammatory, anti-oxidative and chemopreventive properties have been postulated for secondary plant substances such as the group of anthocyanidines (in plants e. g. anthocyanidine glycosides or anthocyanins), a sub-group of flavonols (Schantz, 2011). For example, fruit from lowbush *V. myrtillus* that show a high amount of anthocyanin content, up to 780 mg/100 g fresh weight, are used as natural substances in the prevention and therapy of intestinal diseases (Oehme, 2010). Wild blueberries have a much higher anthocyanin content than cultured blueberry species (for instance *Vaccinium corymbosum* with a content of 180 mg/100 g fresh weight) because the chromophoric anthocyanin is present not only in the pericarp, but also in the flesh of the fruit (Schantz, 2011).

The American official blueberry gene bank (ARS) in Corvallis, Oregon, houses blueberries from the USA and foreign countries in order to safeguard the species for future generations. This shows the importance of this plant species (Wood, 2011).

Significant biotechnology research has been done in the area of micropropagation, specifically in the Canadian berry industry (Debnath, 2007; Debnath, 2009). Strategies for micropropagation of low- and highbush *Vaccinium* species have also been developed in the USA (Shibli and Smith, 1996), the Czech Republic (Paprstein et al., 2004), Slovakia (Ostrolucká et al., 2004; Gajdosovà et al., 2006), Poland (Borkowskka and Krewinska, 2007) and Finland (Jaakola et al., 2000). In Austria a comprehensive master's thesis has been written on the evaluation of native *Vaccinium myrtillus* provenances (Sattler, 2011). Additional work such as habitat characterization, analysis of cultivation procedures for field trials, identification of mycorrhiza strains, and determination of soil parameters (Bohner et al., 2014) and fruit properties have been carried out at five different Austrian institutes (Balas et al., 2012;

Bohner et al., 2012; Gantar et al., 2012) from 2012 to 2015 under the national project "Evaluation of the Fruit Growing Potential Capacity of Autochthonous and Selected Wild Blueberry Ecotypes (*V. myrtillus*)". Significant biotechnology research has been done in the area of micropropagation (Steniczka et al., 2006; Popowich et al., 2007; Gajdoŝovàet al., 2009).

In this paper micropropagation via axillary and adventitious shoots regeneration through adult (mature) and juvenile tissue for Austrian provenances of *V. myrtillus* is described. It is examined whether *in vitro* cultivation is a viable method of propagation.

MATERIAL AND METHODS

ESTABLISHMENT VIA NODE SEGMENTS

Vaccinium myrtillus of eight Austrian habitats were established via node segments between 2011 and 2013 (Table 1). Explant removal took place shortly after bud burst on potted plants (establishment 2011 and 2012) or directly in the habitat itself (establishment 2013). On the June, 21st, 2011 establishment, the Nordwald, Am Hagen and Sailer provenances had only been in pots for a few weeks. Sailer, Planneralm (three collection sites) and Planner/unten, however, had been in pots for one year before establishment on May, 23rd, 2012. The establishment material for 2013 (establishment on May, 23rd, June, 25th, and July, 1st) was taken directly from the habitats at Am Hagen, Nordwald, Kronsegg, Raumberg1 and Raumberg2.

Node segments of 10 to 15 cm freshly sprouted shoots were washed under tap water for 30 to 45 min and sterilized for 19 hours in 1 % PPM™ (Plant Preservative Mixture; Plant Cell Technology, Washington, USA), which had been enriched with 50 mg/l $MgSO_4$ and afterwards were washed with sterilized reverse osmosis water (UO-water) once. An additional overlaying with 0.1 % 8-hydroxy-quinolinol-sulfate (8-HQS) for 24 hours took place during the 2011 establishment (Laimer da Camara Machado et al., 1991).

Nodal segments (21 per provenance in 2011, 9 to 48 in 2012, 40 or 60 in 2013) were cultured in a modified WP medium (0.025 mg/l $CuSO_4$ x 5 H_2O, 0.2 mg/l Thiamin and without K_2SO_4) (Mccown and Lloyd, 1981) with 30 g/l sucrose, 3 g/l gelrite or 1 g/l gelrite and 4 g/l agar, 80 mg/l adenine sulfate or 50 mg/l casein hydrolysate, 2 ml/l PPM™, adjusted to 5.0 pH (Hristoforoglu et al., 2004). Plant growth regulators were used as following: For 2011: 1 mg/l indole acetic acid (IAA) and 5 mg/l N6 (2-isopentenyl)adenine (2iP) (Hristoforoglu et al., 2004); for 2012 and 2013: 1 mg/l IAA and 10 mg/l 2iP or 1mg/l IAA, 1 mg/l gibberellic acid (GA3) and 2 mg/l Zeatin (Hristoforoglu et al., 2004; Gajdoŝovà et al., 2006; Debnath, 2007). Test tubes (16 mm diameter) with 4 ml medium were used for the cultivation of the node segments. 130 ml baby food jars with 40 ml of medium were used for the cultivation of micro-shoots, subcultured in 6 to 8 weeks' intervals. Node culture clones were kept 16 to a jar with between 5 and 15 jars per source. Cultures were maintained at 23 ± 1 °C under cool white-fluorescent light (23 µmol/m²/s), under GROLUX fluorescent light (15 µmol/m²/s) and dim light (0 µmol/m²/s) with a 16 hr photoperiod.

IN VITRO ROOTING

Trials (A to F) for *in vitro* rooting are shown in Table 2. *In vitro* propagated micro-shoots were used for rooting trials, cultured in ECO2-boxes (Duchefa; Haarlem, The Netherlands) equipped with a breathing system (Type L, 3.5 mm filter length). After preceding rooting trials (not all data of the trials A to F are shown in this paper) the following three were further tested: 1 mg/l IBA (indole-3-butyric acid) under 23 µmol/m²/s cool white-fluorescent light; 1 mg/l IBA and 0.8 g/l activated charcoal (Merck) under dim light; 1 mg/l NAA (1-naphthaleneacetic acid) under dim light. The number of micro-shoots per treatment was small at the beginning because of the preceding rooting trials.

EX VITRO ROOTING

In vitro propagated micro-shoots as well as cuttings from acclimatized in vitro plants were pulsed in 1 g/l IBA (conditions the same as for acclimatization) (Debnath, 2007; Debnath, 2009).

Table 1: *In vitro* establishment of nine Austrian *Vaccinium myrtillus*-sites via nodes and/or seeds at different times. Number of explants; number of successfully established and selected (seedlings) *Vaccinium myrtillus* clones

Austrian sites	Date of establishment	Explant	Number of explants	Number and code of established clones
1) Nordwald, 800 m	21-Jun-2011	Node	21	0
	23-May-2013	Node	40	0
2) Am Hagen, 314 m	21-Jun-2011	Node	21	0
	23-May-2013	Node	40	0
3) Sailer, 1100 m	21-June-2011	Node	21	0
	23-May-2012	Node	19	1 (S/3)
4) Planneralm, 1750 m	23-May-2012	Node	22 (1a)	1a) 0
		Node	9 (1b)	1b) 1 (PA/1b/1)
		Node	48 (1c)	1c) 1 (PA/1c/1)
5) Planner/unten, 713 m	23-May-2012	Node	19	1 (PU/1)
6) Kronsegg, 334 m	25-Jun-2013	Node	40	0
	2-Apr-2014	Seeds*	-	0
	29-Jul-2014	Seeds	-	8 (KR/S2, KR/S3, KR/S6, KR/S12, KR/S14, KR/S15, KR/S17, KR/S21)
7) Raumberg1, 1230 m	1-Jul-2013	Seeds	60	0
	2-Sep-2014	Seeds	-	5 (R1/S7, R1/S16, R1/S19, R1/S21, R1/S25)
8) Raumberg2, 780 m	25-Jul-2013	Seeds	60	0
9) Mölbegg, 1200 m	14-Jul-2014	Seeds	-	6 (MÖ/S1, MÖ/S15, MÖ/S22, MÖ/S26, MÖ/S30, MÖ/S31)

*Seeds from August 2013

Table 2: Rooting trials on modified media according to Murashige & Skoog, 1962 (MS) and McCown & Lloyd, 1981 (WPM)

Trial	Medium	PGR and supplements	Light intensity (µmol/m^2/s)
A	Mod. WPM	0.2 mg/l IBA	Light
B	Mod. MS	1.0 mg/l IBA	Light
C	Mod. WPM	1 mg/l IBA	Light/dim light
D	Mod. WPM	1 mg/l IBA, 0.8 g/l activated charcoal	Light/dim light
E	Mod. WPM	1 mg/l NAA	Light/dim light
F	Mod. WPM	10 mg/l IAA	Light/dim light

Light = 23 µmol/m^2/s, cool white fluorescent light; Dim light = 0 µmol/m^2/s; Mod. MS = modified MS medium (0.2 mg/l Thiamin)
Mod. WPM = modified woody plant medium (see Material and Methods)

ACCLIMATIZATION

Rhododendron soil (Gramoflor; Vechta, Germany) mixed with Perlite (2 : 1) was used as substrate. Plantlets were maintained at 23 ± 1 °C under Sylvania GROLUX fluorescent light (28 µmol/m^2/s) (two weeks 12 µmol/m^2/s, afterwards 28 µmol/m^2/s) with a 16 hr photoperiod. The relative humidity was slowly reduced from 95 % to 65 %.

ESTABLISHMENT VIA SEEDS

The Kronsegg, Mölbegg and Raumberg1 provenances were used for the clonal propagation in 2014 (Table 1): 8.5 months old seeds from August 2013 (Kronsegg provenance seeds established April, 2nd, 2014) and seeds from ripe fruit harvested from July to September 2014 (from Mölbegg, Kronsegg, Raumberg1, established July, 14th, 2014, July, 29th, 2014, September, 2nd, 2014) were washed under tap water for 45 min, put into a Nalgene filtration bottle, pretreated in 70 % ethanol (1 min), decontaminated in a 10 % Danclor (with 5 % active chlorine) / Tween20 solution (10 min) and washed five times in autoclaved UO-water (Hristoforoglu et al., 2004). The modified WP medium (see node establishment) was used for cultivation, enriched with 30 g/l sucrose and 3 g/l activated charcoal (Merck) (Hristoforoglu et al., 2004) and 2 ml/l PPM , adjusted to pH 5.0. 20 Petri dishes (90 mm diameter, 35 ml medium) or 12 to 24 baby food jars (130 ml volume, 40 ml medium) were used for cultivation. Seed cultures were maintained at 23 ± 1°C in dim light (0 µmol/m2/s) or under cool white-fluorescent light (23 µmol/m2/s) of a 16 hr photoperiod. The August 2013 seeds, cultured in Petri dishes, were treated additionally after seven weeks with sterile-filtrated GA3 (7.5 mg/l) (Vijay et al., 2012) or put in a refrigerator at 4 °C for 4 weeks (Sedaghathoor, 2007; Baskin et al., 2000; Castro et al., 2012).

MICROPROPAGATION

After *in vitro* germination seedlings were selected and propagated. For clonal propagation micro-shoots of the seedling clones were kept 8 to a jar with a maximum of two jars per source, on same medium and subculture intervals as for micro-shoots from node segments, as mentioned above.

STATISTICS

The statistical calculations were carried out with the SPSS program for Windows, Version 22. The comparison of the means of two independent samples was carried out with the t-Test according to Student. The comparison of more than two independent samples was performed with a simple analysis of variance (ANOVA) The variance homo- or heterogeneity was reviewed with the Levene-Test. In case of variance heterogeneity of ANOVA results, data have been tested in pairs step by step with the t-Test according to Student (SPSS shows results for homogenous and heterogenous variances using the t-Test). A normal distribution of data was shown by the Shapiro-Wilk-Test.

RESULTS AND DISCUSSION

ESTABLISHMENT AND PROLIFERATION VIA NODE SEGMENTS

ESTABLISHMENT 2011

Node segments from mature tissue of the Nordwald, Am Hagen and Sailer provenances established in 2011 showed bud burst in 1 mg/l IAA and 5 mg/l 2iP as follows: provenance Nordwald: 27 %, provenance Am Hagen: 0 % and provenance Sailer: 27 % (Fig. 1; Table 1). Burst buds showed intact or destroyed (only leaf development was visible) shoot meristems. However, both did not show further growth and died after a few weeks. The rest of the cultured nodes died due to fungi, bacteria and/or explant loss. The node segments which had been treated in 0.1 % 8-HQS additionally to the PPM™ decontamination, showed distinctly lower frequencies of bud burst of 0 %, 0 % and 10 % (data not shown in Fig. 1). The high amount of dead node segments (except for Am Hagen with 20 %) on Nordwald with 100 % and Sailer with 60 % might have been caused by the additional treatment with 8-HQS. Loss due to fungi and/or bacteria could not be reduced (Am Hagen with 40 % and 40 %, Sailer with 30 % and 0 % (data not shown in Fig.1). These results are not in agreement with Laimer da Camara Machadeo et al. (1991) who had successfully established a tissue culture from adult apple trees by 8-HQS. In the latter case the infection rate as well as the phenolic compounds could be strongly reduced. In our case the phenolic compounds did not play any role.

ESTABLISHMENT 2012

The Sailer, Planneralm and Planner/unten provenances, established in 2012, showed bud burst in 1 mg/l IAA and 10 mg/l 2iP or 1 mg/l IAA, 1 mg/l GA^3 and 2 mg/l Zeatin, within 6 to 8 or 6 to 18 weeks. Total induction frequencies of 16 %, (18 %, 11 %, 4 %) and 11 % were obtained in both media. Whereas in 1 mg/l IAA and 10 mg/l 2iP only bud burst and slow growth took place, in 1 mg/l IAA, 1 mg/l GA_3 and 2 mg/l Zeatin proliferation and induction of adventitious micro-shoots could be observed. The subclones of Sailer S/3, Planneralm PA/1b/1, Planneralm PA/1c/1 and Planner/unten, PU/1 were successfully established.

ESTABLISHMENT 2013

The Am Hagen, Nordwald, Kronsegg, Raumberg1 and Raumberg2 provenances, established in 2013, showed bud burst (except for Nordwald) on both media as used in 2012. Induction frequencies of 10 %, 0 %, 40 %, 16 % and 2 % could be observed. Same as in the establishment 2011, despite bud burst up to 40 %, any further growth could not be achieved, and the cultivated node explants died within a few weeks.

All in all a successful establishment from cell lines was only possible in 2012. As shown in Figure 1 the percentage of loss (due to bacteria, fungi and explant loss) of node segments in relation to bud burst loss and established subclones was high. The loss due to bacteria was mainly caused by cyanobacteria. They could not successfully be eliminated despite treatments with 100 % of the plant preservative mixture PPM^{MT} (one to two droplets per explant). The slow growth of shoots might have caused the unsuccessful elimination of cyanobacteria. The high frequency of loss due to bacteria or fungi on Raumberg1 and Raumberg2 may have been caused by the late establishment in June/July due to a higher availability of mature plant material at that time. In comparison, on wild *Rhododendron* species, also established via mature shrubs, bud burst could only be initiated on a few genotypes. However, these could be successfully established (Hristoforoglu et al., 2004).
As all three establishments included plant material of adult shrubs, there is one important factor that may be responsible for the successful establishment in 2012, namely the re-cultivation of the *Vaccinium myrtillus* provenances in pots. The established Sailer, Planneralm and Planner/unten provenances had been re-cultivated in pots for one year before establishment, whereas in 2011 the Nordwald, Am Hagen and Sailer provenances had only been in pots for a few weeks. In 2013 the explant removal took place directly in the habitat itself. It can be assumed that preconditioning in pots for 12 months enabled the 2012 establishment. Yet, the successful establishment in 2012 still turned out to be difficult, because the established subclones needed up to 8 months for the induction of adventive micro-shoots. Although it is well known that adult material is difficult to propagate in comparison to juvenile material, its advantage is that plant material (elite clones) improved by breeding and selecting can be very quickly clonally propagated. In this context Debnath (2007) and Preece (2008) mention that the initial explant is strongly influenced by the age of the tissue or organ which is used.

The subculturing of the established provenances took place within 6 to 8 weeks on 1 mg/l IAA, 1 mg/l GA_3 and 2 mg/l Zeatin (Fig. 2). During subcultivation under cool white-fluorescent light (23 µmol/m^2/s) rapid degradation, strong lignification of stems and yellow coloring of the leaves were observed. Throughout the 13 subcultivations no clear increase in mean number of micro-shoots per explant were visible. Only slight differences between clones were observed: Sailer S/3 (1.65 ± 0.26 to 2.37 ± 0.27), Planner/unten PU/1 (1.41 ± 0.17 to 1.98 ± 0.37), Planneralm PA/1b/1 (1.86 ± 0.31 to 1.46 ± 0.12), Planneralm PA/1c/1 (1.32 ± 0.15 to 1.86 ± 0.32) (Fig. 3). Rejuvenation was not observed either. The cultured micro-shoots showed a low stretching and a rapid lignification of sprouts as well as a loss of shoot meristems. This is not in correspondence with *Rhododendron*, where rejuvenation took place during subculturing, which could be seen in a high amount of induced micro-shoots with satisfying growth and hardly any lignification (Hristoforoglu et al., 2004). Trials under GROLUX-fluorescent light (15 µmol/m^2/s) and dim light (0 µmol/m^2/s) did not show any better results. Under dim light the quality of the induced micro-shoots

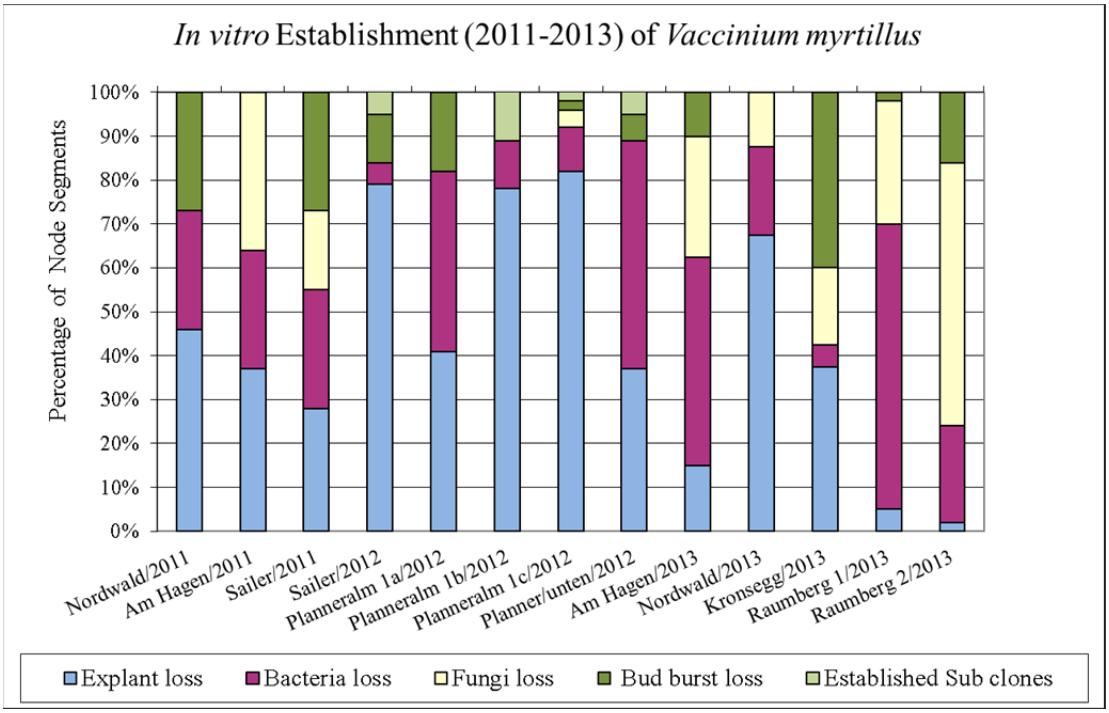

Fig. 1: *In vitro* establishment of *V. myrtillus* provenances via node segments, from 2011 to 2013; relation (%) between explant loss, bacteria loss, fungi loss, bud burst loss and established subclones; the composition of the 2011 medium differs from the ones in 2012 and 2013 by a lower 2iP- concentration (5.0 mg/l instead of 10.0 mg/l).

was better (seen in slower degradation and lower lignification) but during the proliferation intervals they became thinner and thinner, so that for rooting trials only micro-shoots from trials under cool white-fluorescent light were used. The statistical analysis in Figure 3 shows the following results:

Statistical analysis of data revealed a significant impact of the subclones (three provenances) Sailer S/3, Planner/unten PU/1, Planneralm PA/1b/1 and PA/1c/1 on the number of micro-shoots per explant. The subclone Sailer S/3 showed a significantly higher number of micro-shoots per explant (M = 1.84, SD = 0.109) compared with the subclones Planner/unten PU/1 (M = 1.161, SD = 0.150) and Planneralm PA/1b/1 (M = 1.59, SD = 0.162) and PA/1c/1 (M = 1.58), SD = 0.178).

In contrast a significant influence of the times of subcultivation (April, 16th, 2014 to April, 14th, 2015, 8 points of time compared) on the number of micro-shoots was not established.

ESTABLISHMENT AND PROLIFERATION VIA SEEDS

8.5 months old seeds from the Kronsegg provenance did not show any germination under cool white-fluorescent light (23 µmol/m^2/s) or dim light (0 µmol/m^2/s) after cultivation on the WP medium with 3.0 g/l activated charcoal without plant growth regulators. The treatments with GA_3, 6 weeks after cultivation, as well as a four-weeks cooling at 5 °C, could not break the dormancy of seeds either. Compared to this trial, a cultivation of freshly harvested ripe fruit and immediately cultivated seeds from the Kronsegg, Mölbegg and Raumberg1 provenances started to germinate within 10 days under cool white-fluorescent light (23 µmol/m^2/s) (Fig. 4). As the germination took place so rapidly, it is presumed that the seeds had not yet gone dormant. For further studies it would be interesting to find out at what time dormancy starts and how it can be broken *in vitro*. For a

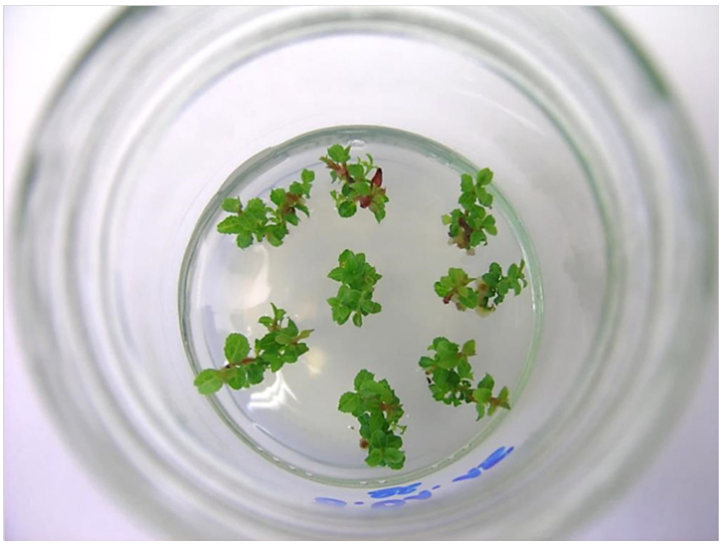

Fig. 2: Micropropagated shoots of *V. myrtillus*, Sailer S/3 provenance in a modified WP medium enriched with 1 mg/l IAA, 1 mg/l GA_3 and 2 mg/l Zeatin five weeks after subculturing; establishment via node segments of adult shrubs

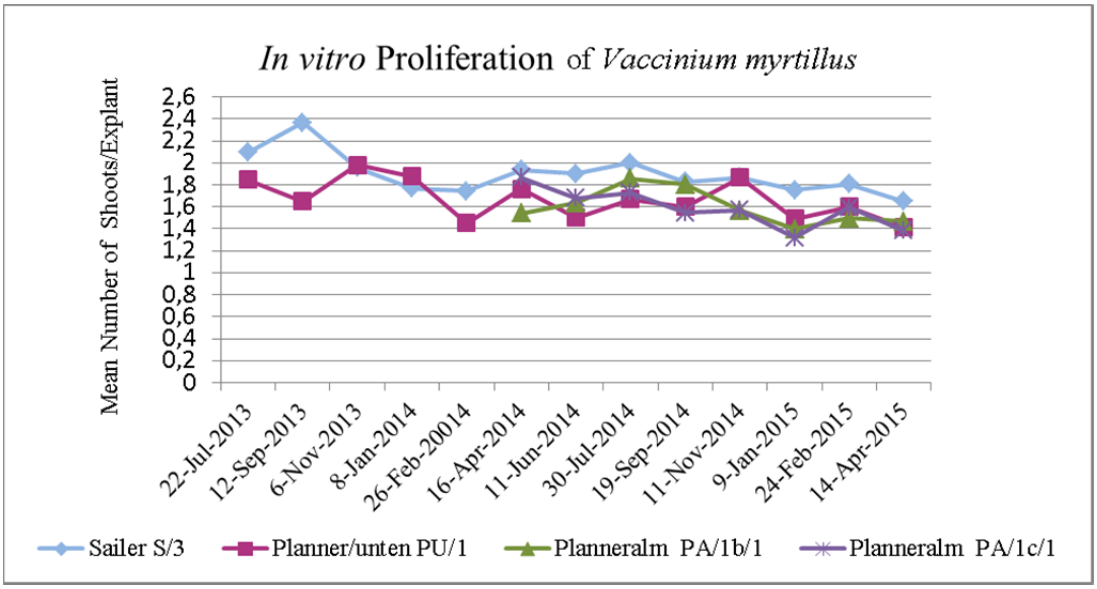

Fig. 3: Mean number of micro-shoots/explant on four *V. myrtillus* subclones (three provenances) during 13 subculturing times; subclones: Sailer S/3, Planner/unten PU/1, Planneralm PA/1b/1 and PA/1c/1; period: July, 22nd, 2013 to April, 14th, 2015; significant statistical differences were observed between Sailer S/3 and Planner/unten PU/1, Planneralm PA/1b/1 and PA/1c/1.

further clonal propagation 30 or 32 seedlings were selected from each provenance. A clear difference can be observed between the proliferation of the node-generated clones and the seedling clones. Whereas the initiation of adventitious shoots on adult material started after 8 months, on juvenile seedlings it took place immediately after the transfer to the propagation medium enriched with 1 mg/l IAA, 1 mg/l GA_3 and 2 mg/l Zeatin. Altogether 92 seedlings were clonally propagated from the Kronsegg, Mölbegg and Raumberg1 sites of which 19 seedling clones were finally selected as plus clones after propagation and rooting trials (Table 1; Table 3). Comparing the propagation of the subclones with the one of the seedling clones, higher induction rates are observed on the seedling clones (Fig. 3; Table 3).

Statistical analysis proved a distinct deviation among the seedling provenances. The mean number of micro-shoots per explant at the low site Kronsegg (M = 3.98, SD = 1.124) (334 m above sea level) was significantly higher (p = 0.000) in comparison with Mölbegg (M = 2.45, SD = 0.649) and Raumberg1 (M = 2.22, SD = 0.693) (1200 m and 1230 m above sea level) (Table 1; Table 3). There was no significant difference between Mölbegg and Raumberg1. The date of subculture of the 19 genotypes, in contrast, had a significant impact on the number of shoots per explant. The micro-shoots per explant at subculture date 2 (M = 2.58, SD = 0.923) were significantly fewer (p = 0,036) compared to those at subculture date 4 (M = 3.52, SD = 1.604).

Within the eight genotypes at Kronsegg no significant difference in formation of micro-shoots was observed. The date of subculture of the eight genotypes, in contrast, had a significant impact on the number of shoots per explant. The micro-shoots per explant at subculture date 2 (M = 3.32, SD = 0.937) were significantly fewer (p = 0,014) compared to those at subculture date 4 (M = 4.93, SD = 1.260).

Within the genotypes at Mölbegg and Raumberg1 in contrast no statistically significant influence of genotype or date of subculture on the number of micro-shoots per explant was found.

Apart from the significantly higher induction rates, the induced micro-shoots of Kronsegg showed a distinctly more juvenile appearance. Taking these results as a basis, it is postulated that sea level has an important influence on propagation, as Mölbegg and Raumberg1 are situated at 1200 and 1230 m, nearly 900 m higher than Kronsegg with 334 m. The influence of the sea level (Sailer 1100 m, Planner/unten 713 m, Planneralm 1750 m) could have been an additional difficulty during the establishment of the node cultures.

Fig. 4: Seedlings from *V. myrtillus*, Kronsegg provenance in a modified WP medium, without growth regulators and enriched with 3.0 g/l activated charcoal, four weeks after cultivation; establishment on July, 29th, 2014 via ripe fruit

Table 3: Selected plus clones from provenances Kronsegg, Mölbegg and Raumberg1, established over seeds. Single (8 to a jar with one jar per source) or mean value (8 to a jar with two jars per source) of micro-shoots per explant of 4 subculture dates. Root frequency (% vital micro-shoots with initiation of ≥ 1 root per micro-shoot) on 8 different trials

Provenance	Selected clones	Number of shoots/ explant				Root frequency (%)
		Date 1	Date 2	Date 3	Date 4	
Kronsegg	KR/S2	3,94 ± 1,09	3,69 ± 0,44	4,69 ± 0,27	5,19 ± 0,27	14[1], 33[2]
	KR/S3	5,38 ± 0,35	2,44 ± 0,62	4,75 ± 0,18	5,81 ± 0,44	58[1]
	KR/S6	2,89	4,0 ± 1,06	3,25 ± 0,53	5,00 ± 0,35	0[2], 20[4]
	KR/S12	3,38	2,13	3,31 ± 0,09	2,31 ± 0,44	50[4], 90[6]
	KR/S14	3,50	3,94 ± 0,27	3,06 ± 0,27	5,13 ± 0,18	6[2], 71[4]
	KR/S15	3,0 ± 0,85	4,19 ± 0,27	5,06 ± 0,27	5,50 ± 0,18	36[1], 20[4]
	KR/S17	3,13	3,63	3,06 ± 0,09	4,94 ± 0,62	45[2], 47[4]
	KR/S21	3,75	2,56 ± 0,44	5,0 0± 0,71	4,75 ± 0,18	38[2], 31[6]
Mölbegg	MÖ/S1	2,63	1,44 ± 0,80	2,31 ± 0,09	2,06 ± 0,62	0[2], 0[4],10[7]
	MÖ/S15	2,13	2,38	3,25 ± 0,18	2,63 ± 0,35	33[7]
	MÖ/S22	3,50	2,00	3,56 ± 0,62	2,25 ± 0,18	33[7]
	MÖ/S26	2,33	3,13	2,81 ± 0,09	3,25 ± 0,71	43[7]
	MÖ/S30	2,88	1,94 ± 0,27	1,50 ± 0,35	2,13 ± 0,71	40[7]
	MÖ/S31	1,83	2,13	2,19 ± 0,80	3,00 ± 0,35	14[7]
Raumberg1	R1/S7	2,80 ± 0,12	2,13 ± 0,18	4,25 ± 0,71	4,62 ± 0,53	19[3], 52[8]
	R1/S16	2,50	2,06 ± 0,09	2,94 ± 0,44	1,67 ± 0,12	33[5], 29[8]
	R1/S19	2,00	2,21 ± 0,83	1,50 ± 0,53	2,32 ± 0,26	9[5], 0[8]
	R1/S21	2,00	2,06 ± 0,44	2,00 ± 0,71	2,32 ± 0,09	0[5], 7[8]
	R1/25	1,71	1,88 ± 0,18	2,18 ± 0,09	2,01 ± 0,09	42[5], 60[8]

Subculture dates: 1: 14 Jan 2015 to 11 Feb 2015, 2: 4 Mar 2015 to 1 Apr 2015, 3: 22 Apr 2015 to 18 May 2015, 4: 18 Jun 2015 to 8 Aug 2015
[1] Trial 26 Jan 2015, [2] Trial 16 Mar 2015, [3] Trial 14 Apr 2015, [4] Trial 4 May 2015, [5] Trial 2 Jun 2015, [6] Trial 25 Jun 2015, [7] Trial 1 Jul 2015, [8] Trial 16 Jul 2015
Significant statistical differences were observed between Kronsegg/Mölbegg and Kronsegg/Raumberg1 sites. Significant statistical differences were observed between subculture date two and four (for Kronsegg itself and for all sites together)

ROOTING AND ACCLIMATIZATION OF PLANTLETS ESTABLISHED VIA NODE SEGMENTS

After testing the media listed in Table 2 the following modified media were selected according to Mccown and LLOYD (1981): see Table 2, trial C: 1 mg/l IBA (23 µmol/m²/s, cool white-fluorescent light), trial D: 1 mg/l IBA and 0.8 g/l activated charcoal (0 µmol/m²/s, dim light) and trial E: 1 mg/l NAA (0 µmol/m²/s, dim light). On Sailer and Planner/unten a time slot (rooting-trials: June, 12th, 2013, July, 22nd, 2013, September, 11th, 2013) could be observed in 1 mg/l IBA or 1 mg/l NAA, in which distinctly higher rooting frequencies were achieved (Fig. 5; Fig. 6). While with Sailer 60 % (3 out of 5), 67 % (6 out of 9) and 83 % (15 out of 18) of the micro-shoots developed roots, with Planner/unten rooting frequencies of 80 % (4 out of 5), 89 % (8 out of 9), 89 % (16 out of 18) and 100 % (9 out of 9) could be achieved. In 1 mg/l NAA both provenances showed one high frequency peak on July, 22nd, 2013, whereas in 1 mg/l IBA on Sailer two (June, 12th, 2013, July, 22th, 2013) and on Planner/unten, three high frequency peaks (June, 12th, 2013, July, 22nd, 2013, September, 11th, 2013) could be observed, respectively. At the remaining rooting times rooting rates of ≤ 38 % (9 out of 24) were achieved. There were additional interesting results with Sailer in 1 mg/l IBA enriched with 0.8 g/l activated charcoal. Distinctly higher rooting frequencies were achieved: 67 % (6 out of 9), 100 % (9 out of 9), 89 % (8 out of 9), 67 % (6 out of 9), 56 % (30 out of 54), 75 % (27 out of 36) and 90 % (27 out of 30) during the trials on September, 11th, 2013, November, 6th, 2013, January, 7th, 2014, February, 25th, 2014, June, 12th, 2014, November, 10th, 2014 and April, 14th, 2015, respectively. The highest peaks in both 2013 and 2014 were achieved in November, with 75 % (27 out of 36) in 2014 and 100 % (9 out of 9) in 2013. Upon closer examination of the Planner/unten provenance in

activated charcoal, the positive effect of the activated charcoal was not really visible in the first trials, but a clear increase in the rooting rates became apparent after July, 29th, 2014 (56 % (5 out of 9) rooting frequency). Due to the obvious boosting effect of the activated charcoal on the root development, the Planneralm (subclone PA/1b/1, subclone PA/1c/1) provenance were only rooted in 1 mg/l IBA enriched with 0.8 g/l activated charcoal. The subclone Planneralm PA/1b1 had clearly higher rooting frequencies (June to September 2014: 59 % (16 out of 27), 81 % (29 out of 36) and 94 % (34 out of 36)) in comparison with the subclone Planneralm PA/1c/1 (22 % (6 out of 27), 19 % (7 out of 36) and 44 % (16 out of 36)), seen in Figure 7. Looking at the result of all three provenances (Sailer S/3, Planner/unten PU/1, Planneralm PA/1b/1 and PA/1c/1 subclones) in the charcoal medium, the lowest rooting rates for 2014 all occurred on April, 14th (Fig. 7). After an increase (June, 12th, to the highest peak on September, 17th, with the exception of Sailer on November, 10th) a repeated reduction could be observed. Compared to 2014, in 2015 the lowest rooting rates were observed in February (except for Planneralm PA/1c/1 in January) and started again to increase in a steeper curve than in 2014. These results are not comparable with the ones from *Rhododendron*, where successful rooting on 0.2 mg/l IBA was achieved during the entire year (Hristoforoglu et al., 2004). Can this phenomenon be attributed to the endogenous physiological rhythm of the established adult plant material? The detailed statistical analysis in Figure 7 shows the following results:

In order to be able to review the success of rooting (1 = 100%, 0 = 0%) in relation to time, the following method was applied: the possible influence of the four subclones (Sailer S/3, Planner/unten PU/1, Planneralm PA/1b/1 and PA/1c/1) was not considered in this calculation and n = 4. Nine common, different rooting times were compared with each other (February, 25th, 2014 to April, 14th, 2015). The rooting time of April, 15th, 2014 differed significantly from the time of rooting on September, 17th, 2014 ($p = 0.022$), on November, 10th, 2014 ($p = 0.039$) and on April, 14th, 2015 ($p = 0.013$). At the time of rooting on April, 15th, 2014 there was a significantly lower rooting ($M = 0.07$, $SD = 0.069$) compared with the rooting time on September, 17th, 2014 ($M = 0.63$, $SD = 0.231$), November, 11th, 2014 ($M = 0.59$, $SD = 0.181$) and April, 14th, 2015 ($M = 0.66$, $SD = 0.219$). In order to review the success of rooting in relation to the four subclones, the influence of the nine different rooting times was not considered, and n = 9. There was a significant difference ($p = 0.012$) between Planneralm PA/1b/1 and PA/1c/1. Planneralm PA/1b/1 ($M = 0.60$, $SD = 0,248$) showed a significantly higher success in rooting in comparison to Planneralm PA/1c/1 ($M = 0.26$, $SD = 0.173$).

In comparison with the *in vitro* rooting trials, the direct rooting of micro propagated shoots in *Rhododendron* soil (Gramoflor; Vechta, Germany)/Perlite (2 : 1), after a 1 g/l IBA pulse treatment, did not achieve better results. A large number of losses occurred during rooting period of up to three months. This is not in agreement with Debnath (2007; 2009) who found 85 to 95 % rooting in lowbush blueberries when 3 to 4 cm long *in vitro* derived shoots were dipped into 39.4 mM IBA before planting. Maybe the loss was caused by short micro-shoots of < 15 mm. Satisfying and surprising rooting results could be achieved after a 1 g/l IBA pulse treatment (November, 4th, 2014, December, 9th, 2014, January, 13th, 2015) on 20 to 40 mm long cuttings from in *Rhododendron* soil (Gramoflor; Vechta, Germany)/Perlite (2 : 1) acclimatized *in vitro* plants (two to four months old) from the Sailer S/3 and Planneralm PA/1b/1 provenances. 67 % (6 out of 9) and 100 % (35 out of 35) as well as 100 % (23 out of 23) and 84 % (27 out of 32) rooted within three to four weeks. The growth was definitely better than that of the plantlets rooted *in vitro*. Branching started about 6 to 8 weeks after the IBA pulse treatment, earlier than with in vitro rooted plants after acclimatizing. Thus, first observations did not show any distinct differences as seen on softwood stem cuttings in comparison to micropropagated plants, namely fewer basal branches and lateral shoots described by Debnath (2007).

Acclimatized plantlets of the three provenances (four subclones) from i*n vitro* rooting trials showed a distinct difference in their growth. While growth started after two weeks on the Sailer S/3 and Planneralm

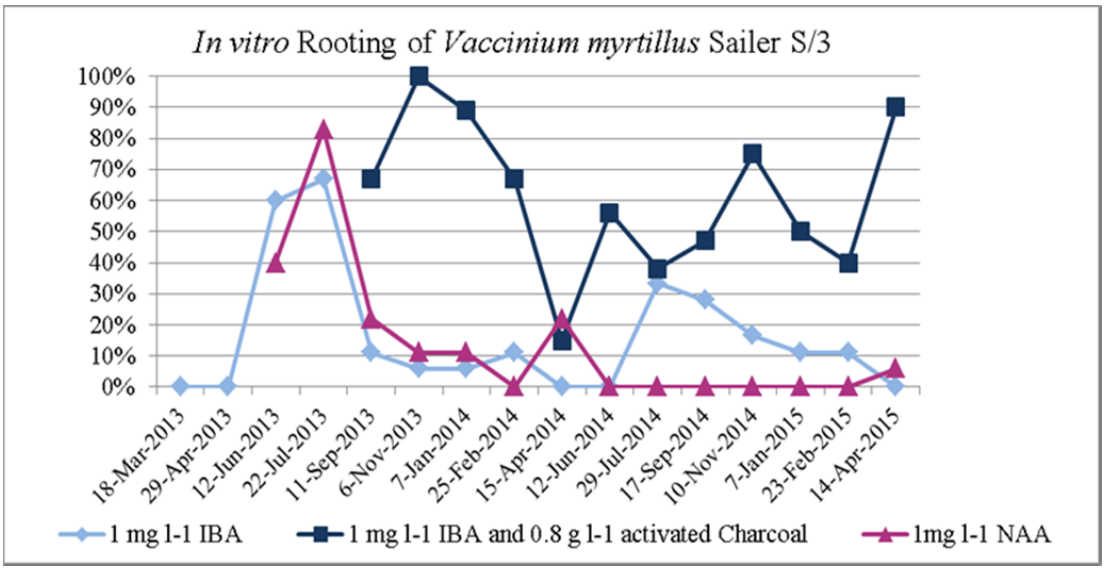

Fig. 5: Percentage of root development (percentage of vital micro-shoots with initiation of ≥ 1 root per micro-shoot) on *V. myrtillus* Sailer S/3 provenance during 16 rooting times, from March, 18th, 2013 to April, 14th, 2015; plant growth regulators: 1 mg/l IBA, 1 mg/l IBA and 0.8 g/l activated charcoal, 1 mg/l NAA

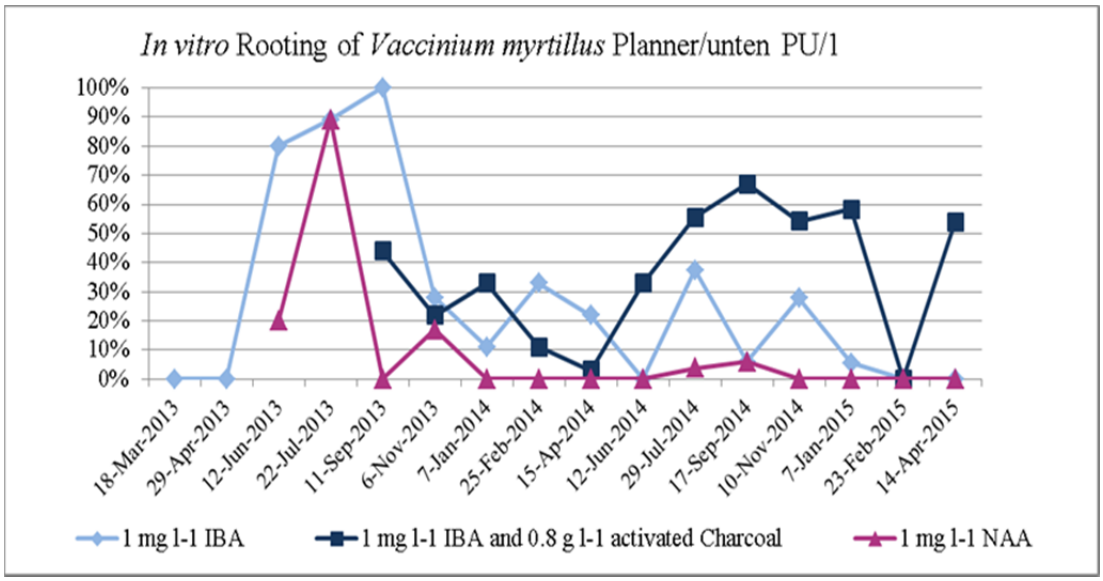

Fig. 6: Percentage of root development (percentage of vital micro-shoots with initiation of ≥ 1 root per micro-shoot) on *V. myrtillus* Planner/unten, PU/1 provenance during 16 rooting dates from March, 18th, 2013 to April, 14th, 2015; plant growth regulators: 1 mg/l IBA, 1 mg/l IBA and 0.8 g/l activated charcoal, 1 mg/l NAA

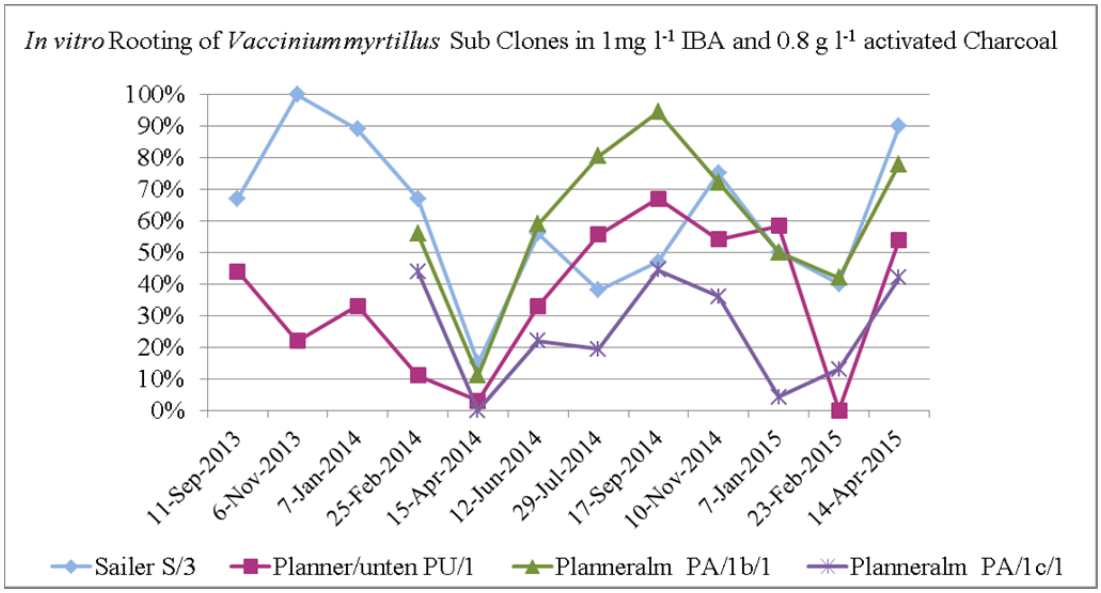

Fig. 7: Percentage of root development (percentage of vital micro-shoots with initiation of ≥ 1 root per micro-shoot) in 1 mg/l IBA and 0.8 g/l activated charcoal, during 12 rooting times from September, 11th, 2013 to April, 14th, 2015; *V. myrtillus* subclones: Sailer S/3, Planner/unten PU/1, Planneralm PA/1b/1 and PA/1c/1

Significant statistical differences were observed between the rooting times of April, 15th, 2014/ September, 17th, 2014, April, 15th, 2014/November, 10th, 2014 and April, 15th, 2014/April, 14th, 2015. Significant statistical differences were observed between the subclones Planneralm PA/1b/1 and PA/1c/1.

PA/1b/1 subclones, on Planner/unten PU/1 and Planneralm PA/1c/1 only on a few plants length-growth could be observed. Sailer S/3 and Planneralm PA/1b/1 developed basal branches and lateral shoots after two months and grew vigorously (Fig. 8; Fig. 9). On Planner/unten PU/1 and Planneralm PA/1c/1, repeated necrosis on shoot meristems with new sprouting was observed, so that at the end only a few plantlets survived. The strong differences among and within the provenances might have been dependent on genotype. Thus it is meaningful to establish a stock of mother plants for direct rooting over cuttings as they enable a rapid, efficient and easy reproduction of *V. myrtillus* plantlets.

ROOTING AND ACCLIMATIZATION OF PLANTLETS ESTABLISHED VIA SEEDS

Interesting results could be achieved in the *in vitro* rooting of the 19 selected seedling clones (Kronsegg, Mölbegg, Raumberg1 provenances) in the WPM with 1 mg/l IBA enriched with activated charcoal. The hypothesis that it would be possible to achieve higher and more reproducible rooting rates on juvenile plant material was not confirmed. In contrast to the clones from node cultures, big differences within and among the provenances could be seen (Table 3). Using the example of the Kronsegg provenance, clear differences in the rooting frequencies could be achieved within the seedling clones KR/S12 of 50 % (3 out 6) and 90 % (19 out 21) and KR/S14 of 6 % (1 out of 18) and 71 % (12 out of 17). In order to talk about a time slot as in the case of the clones of the node culture, more extensive trials would have been necessary. In contrast to the clones of the node culture it was possible to achieve the rooting of *in vitro* micro-shoots after a 1 g/l IBA pulse treatment. Additionally, successful direct rooting from cuttings from juvenile, acclimatized micropropagated plants was achieved. Plantlets from all 19 selected seedling clones were acclimatized for the field trial.

Fig. 8 and 9: Acclimatized *V. myrtillus in vitro* plants of the Sailer S/3 provenance, four months after acclimatization, potted in LIECO-container L40 with Rhododendron soil (Gramoflor; Vechta, Germany) mixed with perlite; acclimatization room under 12 to 28 µmol/m²/s Sylvania GROLUX fluorescent light, 23 ± 1 °C and a relative humidity of 95 % slowly reduced down to 65 %

In summary, when using adult (mature) establishment material, preconditioning in pots for a minimum of 12 months proved to be necessary. When establishing via seeds, the age of the seeds played an important role. Seeds from ripe harvested berries germinated *in vitro* only if they were cultivated immediately after harvesting. During propagation the induction of micro-shoots on the seedling clones took place more rapidly than on the clones of the adult material. During *in vitro* rooting there were significant differences in the rooting rates because of the time of taking the material, which might have been caused by endogenous physiological factors of the various genotypes. However, the rooting of cuttings from 2 to 4 months old acclimatized plants from *in vitro* propagation proved to be extremely promising and easy to replicate.

For further studies the *in vitro* produced *Vaccinium myrtillus* plants of the Sailer, Planner/unten, Planneralm (PA/1b/1), Kronsegg, Mölbegg and Raumberg1 provenances will be tested for yield parameters in field trials (HBLFA Raumberg-Gumpenstein, etc.).

ACKNOWLEDGEMENTS

This project was financially supported by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW), DAFNE-Nr.100878.
The authors gratefully acknowledge the cooperation among the five participating Austrian institutes (HBLA und BA Klosterneuburg, HBLFA Raumberg-Gumpenstein, HBLFA Schönbrunn, BOKU, OWZ Kärnten). Thanks to Maria Anna Steinbauer for translation and proof reading and Tristan Deppe for proofreading. Thanks to the reviewers for their valuable suggestions.

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Received December, 18th, 2015