Micropropagation of rhubarb with special reference to weaning stage and subsequent growth

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Micropropagation of rhubarb (*Rheum rhabarbarum* L.) on MS medium with sucrose 20 g/l and agar 8 g/l, supplemented with benzylaminopurine (BAP) 1 mg/l and indolebutyric acid (IBA) 1 mg/l for initiation and multiplication, was studied with cv. Victoria and clone AF. With clone AF, the effects of rooting method (direct rooting, direct rooting with Floramon A or *in vitro* rooting) and propagule size (height 1.5–2.0, 2.1–5.0 or 5.1–10.0 cm) on weaning survival and plant size were examined. Further growth was recorded over a 3-year period in field studies.

The medium gave a multiplication rate of 3.2/4 weeks for clone AF. For cv. Victoria, the medium did not seem suitable because of high occurrence of callus and vitrification. The multiplication rate of cv. Victoria was, however, increased from 2.8 to 5.4/4 weeks by using propagules from non-sprouting instead of sprouting buds. The weaning survival of clone AF averaged 86%. Rooting method did not affect either weaning survival or plant size. Propagule size affected plant size, but not weaning survival or further growth in the field.

Key words: direct rooting, *in vitro* rooting, growth in field studies, propagule size

Introduction

Rhubarb is usually propagated vegetatively since seed propagation results in undesired variation among progeny. Conventional propagation by crown division gives, at best, a 4- to 6-fold multiplication rate every 2 years. By using single-bud division, ca. 240 plants/mother plant can be produced during a 6-month season (CASE 1970, NORMAN 1978). Viruses are, however, transmitted through these propagation methods. WALKEY (1968) succeeded in eliminating viruses from rhubarb through *in vitro* culture of meristem tips. The method has been further developed for rapid multiplication (ROGGEMANS and CLAES 1979, WALKEY and MATTHEWS 1979, PIERIK et al. 1989, CAMARA MACHADO et al. 1990, RUMPUNEN 1990). In theory, based on the average multiplication rate of 2.8/2 weeks obtained by WALKEY and MATTHEWS (1979), it is possible to produce over a million rhubarb plants within 7 months from a single meristem tip initiated *in vitro*.

In micropropagation, direct rooting is recommended whenever possible because of considerably lower costs compared with *in vitro* rooting. Furthermore, roots formed in vitro are easily dam-

Abbreviations: MS = MURASHIGE and SKOOG (1962), BAP = benzylaminopurine, IBA = indolebutyric acid, NAA = naphthaleneacetic acid

aged during planting (CONNER and THOMAS 1981, DEBERGH and MAENE 1981). To maximize weaning survival, it appears that the propagule must exceed a certain minimum size (CONNER and THOMAS 1981). The leaves formed *in vitro* are found to act mainly as storage organs (WARDLE et al. 1983) and, after transferring to soil, enough nutrient reserves should be present to supply the plant's requirements until total autotrophy is reached (CAPELLADES et al. 1990).

The aim of this study was to investigate micropropagation of rhubarb, especially the effects of direct rooting and propagule size on weaning survival and *ex vitro* growth. To observe the after effects, further growth was examined in a field trial over 3 years.

Material and methods

Clone AF was used as plant material in Experiments 1 and 4, and cv. Victoria in Experiments 2 and 3. In Experiment 1, the shoot multiplication rate was calculated from 139 propagules and in Experiment 2 from 70 propagules in the third subculture. In Experiment 1, the buds available were mostly non-sprouting and in Experiment 2 mostly sprouting. In Experiment 3, the multiplication rates of propagules from sprouting and non-sprouting buds (13 and 24 propagules, respectively) were compared in the first subculture.

In Experiment 4, where the shoots of clone AF from Experiment 1 were used, the effects of rooting method (*in vitro* rooting, direct rooting and direct rooting with Floramon A treatment (0.1% NAA)) and propagule size (small, medium and large) on weaning survival and plant size *ex vitro* were studied. The propagule size categories, measured as shoot height on the basis of the longest petiole, were 1.5–2.0 cm (small), 2.1–5.0 cm (medium) and 5.1–10.0 cm (large). In Experiment 4, the factor combinations were arranged in triplicate (with 10 plants per replicate) according to a randomized block design.

Experiments 1 and 2 began with *in vitro* initiation in May and Experiment 3 in October 1989. The excised buds from parental crowns were washed in tap water, disinfected in 3.5% sodium hypochlorite for 10 min and rinsed 3 times in sterilized, deionized water. The excised shoot tips were 1–2 mm in diameter. The basal medium consisted of MS medium with sucrose 20 g/l and Bacto agar (Difco) 8 g/l, with pH adjusted to 5.5. For initiation and multiplication, the media were supplemented with BAP (Sigma) 1 mg/l and IBA (Merck) 1 mg/l. No growth regulator was added to the rooting medium. All media were autoclaved for 15 min at 121°C.

The explants were initially grown in culture tubes containing 10 ml medium and, after 4–6 weeks, were transferred to 100 ml Erlenmeyer flasks containing 50 ml medium. The multiplication stage was started after an initiation period of 8 weeks (Experiments 1 and 2) or 6 weeks (Experiment 3). The temperature in the growth chamber was 22–27°C, except during the multiplication stage in Experiments 1 and 2, when it occasionally rose to 31°C. Daylength was 16 h and irradiance ca. 6 W m^{-2} (PAR) (lamp type 'Kirkas de Luxe', Airam).

In Experiment 4, the shoots for *in vitro* rooting were kept for one week on the rooting medium. At transplanting, 32% of the shoots had 1–10 visible roots 0.1–5.0 cm in length. The shoots for direct rooting were harvested directly from the multiplication medium, and half of the shoots were treated with Floramon A. For transplanting, all shoots were transferred to a glasshouse and placed in a plastic tent which was shaded during sunny days. The growth substrate was a 2:1 mixture of fertilized peat (Vapo B2) and vermiculite (grain size 2–3 mm). The mean air temperature in the glasshouse was 22°C. Natural light conditions prevailed at the start of the growing period (September), and after 5 weeks supplementary light was provided for 16 h/day (lamp type HPI-T, Philips). After 18 days, the relative humidity was reduced from 100% to ca. 70% over a period of one week by gradually opening the tent. At the same time, the irrigation water was supplemented with gradually increasing amounts (0.05–0.2%) of fertilizer. During the first 3 weeks, the fertilizer was '4-Superex' (17N–4P–25K, Kekkilä), and thereafter '5-Superex' (11N–4P–25K, Kekkilä).

Plants rooted *in vitro* and those rooted directly without Floramon A (all size categories: 2 x 3 x 8 = 48 plants) were potted and overwintered in a glasshouse until May 1990 when they were planted randomly in an outdoor nursery at a density of 30 cm x 30 cm. On 29 May 1991, the plants were transplanted in a field at a density of 100 cm x 125 cm. Conventional growing techniques were adopted. The first crop was harvested in 1992. The field experiment was arranged in quadruplicate (with two plants per replicate) according to a randomized block design.

In Experiments 1 to 3, the number of shoots/propagule was counted after 4 weeks. Callus occurrence was recorded in Experiments 1 and 2, and vitrification in Experiments 1 to 3. The multiplication data from Experiment 3 were analyzed by Mann-Whitney's U-test. In Experiment 4, the proportion of surviving plantlets, the length of the longest petiole, the number of leaves, and fresh and dry weights of the foliage were recorded. In the field trial, the number of leaves, and the length and the weight of the longest petioles at harvest were recorded. A logistic regression model and analysis of variance were applied to the survival results. The other data were analyzed using analysis of variance and the S–N–K test for mean separation.

Results

In Experiment 1 with clone AF, the multiplication rate was 3.2/4 weeks, and in Experiment 2 with cv. Victoria 2.1/4 weeks. During initiation, callus occurrence was 30% for clone AF and 80% for cv. Victoria, and during the multiplication stage 78 and 100%, respectively. No vitrification was found in clone AF, but in cv. Victoria, 15% of the shoot clusters were vitrified. In Experiment 3 with cv. Victoria, the multiplication rate was higher ($p \leq 0.05$) for propagules from non-sprouting buds (5.4/4 weeks, variation interval [1,17]) than from sprouting buds (2.8/4 weeks, [1,8]). In both treatments, 35% of the shoot clusters were vitrified.

In Experiment 4, no significant differences in weaning survival or plant size caused by different rooting methods were found (Tables 1 and 2). Propagule size did not affect weaning survival significantly, but plant size was affected (Table 1). After a 7-week growing period, plants from large propagules had a dry and fresh weight 4.5-fold higher than those from small propagules (Table 2).

Except for one of the *in vitro* rooted plant of the category "small" that died during the nursery stage, no further plants died later in the experiment. In the field trial, no after effects of the

Table 1. Effect of rooting method and propagule size on weaning survival and plant size after a 4-week growing period in Experiment 4 with clone AF.

Treatment	Weaning survival, %	Petiole length, cm	Number of leaves
Rooting method:			
direct rooting	86.7 a	6.9 a	4.9 a
direct rooting + Floramon A	83.3 a	5.6 a	4.3 a
in vitro rooting	88.9 a	8.0 a	4.7 a
Propagule size:			
small	80.0 a	4.2 c	4.1 b
medium	87.8 a	6.5 b	4.4 b
large	91.1 a	9.8 a	5.2 a

Values for rooting method and propagule size separately followed by the same letter are not significantly different at P = 0.05.

Table 2. Effect of rooting method and propagule size on plant size after a 7-week growing period in Experiment 4 with clone AF.

	Petiole length, cm	Number of leaves	Foliage fresh weight, g	Foliage dry weight, g
Rooting method:				
direct rooting	14.0 a	7.4 a	16.6 a	0.88 a
direct rooting + Floramon A	12.4 a	6.6 a	11.9 a	0.68 a
in vitro rooting	14.8 a	7.1 a	19.0 a	0.96 a
Propagule size:				
small	11.2 c	6.3 b	6.0 c	0.31 c
medium	13.3 b	7.2 a	13.6 b	0.79 b
large	16.6 a	7.7 a	28.0 a	1.41 a

Values for rooting method and propagule size separately followed by the same letter are not significantly different at P = 0.05.

Table 3. Effect of propagule size on the further development and yield of clone AF during 1990–1992.

Year and date	Variable	Propagule size					
		small	CV%	medium	CV%	large	CV%
1990							
21 June	Number of leaves	3.3	39	3.1	32	3.3	24
8 Aug.	" "	13.2	40	15.3	35	15.6	39
1991							
23 May	Number of leaves	24.2	16	26.8	23	26.7	23
	Longest petiole, cm	24.4	15	24.4	20	24.8	13
1992 x)							
22 May	Petiole						
	– length, cm	34	9	34	5	35	4
	– weight, g	100	18	108	10	100	14
4 June	Petiole						
	– length, cm	45	9	45	5	46	5
	– weight, g	121	13	122	14	128	15

No significant differences at P = 0.05.
CV = coefficient of variation for respective variable in CV%.
x) Five largest leaves per plant were harvested on both dates.

rooting method were found (data not presented). The differences between propagule size categories were not significant (Table 3). Variation in the measured growth parameters between individuals was considerable. Among the 47 surviving plants, no morphologically aberrant ones were found.

Discussion

The nutrient medium gave an acceptable multiplication rate for clone AF. However, for cv. Victoria, the medium did not seem optimal for micropropagation because of vitrification and the low multiplication rate in Experiment 2. The dif-

ference in multiplication rate might be due to genotype since cv. Victoria propagates slowly *in vivo* (WALKEY and MATTHEWS 1979). In Experiment 3, the multiplication rate of cv. Victoria was increased by using propagules from non-sprouting instead of sprouting buds. The results of Experiments 2 and 3 are, however, not directly comparable since the time of year for bud excision can affect their hormone and carbohydrate levels (GEORGE and SHERRINGTON 1984).

The high callus occurrence especially in cv. Victoria indicates a too high auxin level, which agrees with the findings of CAMARA MACHADO et al. (1990) with cv. Holsteiner Blut. The occasionally high incubation temperature in the multiplication stage possibly enhanced callus formation and reduced the multiplication rate since the efficiency of cytokinins is found to decrease as the temperature rises, but that of auxins to increase (GEORGE and SHERRINGTON 1984). Further, less callus formation and a higher multiplication rate have been obtained in rhubarb by using glucose instead of sucrose as carbohydrate source (RUMPUNEN 1990).

In a preliminary test with rhubarb, direct rooted shoots survived well but grew more slowly than *in vitro* rooted ones (RUMPUNEN 1990). In our study with clone AF, direct and *in vitro* rooting gave nearly identical results. The small size of the propagules was no obstacle to good survival, but 7 weeks after transplanting, plants from small propagules were clearly inferior in weight to plants from larger propagules. They also had fewer leaves but, at the nursery stage, the number of leaves was almost the same in all propagule categories.

The risk of genetic changes is minimal if shoot tip explants and optimum nutrient medium are used (GEORGE and SHERRINGTON 1984). In our field trial, no morphologically aberrant plants were observed but, between individuals, the variations in number of leaves and petiole length were considerable. These variations could be due to environmental factors. Somaclonal variation cannot be excluded, but since the variations decreased with time, they would most likely be of epigenetic character, possibly associated with virus elimination.

In conclusion, micropropagated shoots of clone AF can be rooted directly, and even shoots 1.5 to 2 cm in height do root and develop well. For cv. Victoria, a suitable initiation and multiplication medium should be designed.

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SELOSTUS

Raparperin mikrolisäys, versojen juurtuminen karaisuvaiheessa ja myöhempi kasvu

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Tutkimuksessa haluttiin selvittää, onnistuuko mikrolisätyn raparperin suora juurrutus turve-vermikuliittialustalle, onko eri pituisten versojen juurtumisessa eroja ja ilmeneekö myöhemmässä kasvussa juurrutustavasta tai lisäyksessä käytetyn verson koosta johtuvia eroja.

Lajiketta Victoria ja kloonia AF mikrolisättiin juurakon silmujen kärkisolukoista Murashigen ja Skoogin kehittämällä alustalla (MS-alusta), joka sisälsi 20 mg/l sakkaroosia ja 8 mg/l agaria. Aloitus- ja monistusvaiheissa alustaan lisättiin 1 mg bentsyyliaminopuriinia ja 1 mg indolivoihappoa litraa kohti. Juurrutusmenetelmän ja verson koon vaikutusta juurtumiseen ja myöhempään kasvuun tutkittiin käyttäen kloonia AF. Tämän kokeen taimien jatkokasvua avomaalla seurattiin kolmen vuoden ajan.

Klooni AF tuotti keskimäärin 3,2 versoa 4 viikossa. Lajikkeella Victoria tulos oli heikompi, ja kallusmuodostusta ja vesisoluisuutta esiintyi runsaasti. Victoria lajikkeen versojen tuotto parantui, kun lisäys tapahtui levossa olevista silmuista. Suoraan turve-vermikuliittialustalle istutetut kloonin AF versot juurtuivat yhtä hyvin (87 %) kuin ne, joiden istutusta edelsi juurrutusvaihe MS-alustalla (89 %). Pienet versot (pisimmän lehden korkeus 1,5 – 2 cm) juurtuivat lähes yhtä hyvin kuin keskikokoiset (2,1 – 5 cm) tai suuret (5,1 – 10 cm). Suurimmista versoista kehittyneet taimet olivat karaisuvaiheen päättyessä kookkaimmat. Taimisto- ja peltoviljelyvaiheissa ei havaittu juurrutustavasta tai verson koosta johtuvia eroja.