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Effects of Age of In Vitro-Derived Potato Plantlets on Early Above- and Below-Ground Development After Planting in Different Cultivars

W. J. M. Lommen[1]

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Abstract
In vitro-propagated potato plantlets are commonly used in potato seed tuber production. Four experiments were carried out to identify how the duration of the last in vitro phase ('age') before planting of in vitro-produced plantlets affects the early growth and development ex vitro, assessed 10–14 days after planting. Experiments included varying ranges in age of the in vitro plantlets at planting (10–40, 15–45, 14–28 and 14–56 days old at the moment of planting in the respective experiments) and different cultivars. Because in vitro plantlet size increases with age, the first experiment studied interactions between age and planting method. Planting 'deep' (the upper four visible leaves above the ground) seemed more robust than planting 'shallow' (all nodes/leaves above ground except the lowest two), and in later experiments, only deep planting was applied. Across experiments, plants grown from younger in vitro plantlets had smaller leaf areas 10–14 days after planting than those from older in vitro plantlets. The increase in leaf area levelled off with increasing age of the in vitro plantlets used, especially when plantlets were older than c. 28 days. Larger leaf areas 10–14 days after planting were related to the older in vitro plantlets having higher absolute growth rates after planting and a larger above-ground leaf area at planting, even though four leaves were kept above ground in all treatments. Below ground, plants from older in vitro plantlets had more stem nodes, initiated stolons earlier after planting and initiated more stolons per plant. Tuber initiation rarely occurred within 14 days after planting, but was observed in some plants from 42- and especially 56-day-old in vitro plantlets of the very early cultivar Gloria and a single plant from 56-day-old in vitro plantlets of the mid-early cultivar Bintje. The data show that planting older in vitro plantlets can enhance early leaf area growth. Plants from older in vitro plantlets also more readily initiate stolons below ground and may be more advanced in the tuber formation process, especially when from early cultivars.

Keywords Acclimatisation · Growth rate · Leaf area · Microplant · Minituber · Node · Normalisation · Planting · *Solanum tuberosum* L. · Stolon initiation · Transplant

Extended author information available on the last page of the article

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Introduction

In potato, in vitro-produced plantlets serve as basic material for the production of several generations of seed tubers. The in vitro plantlets are first multiplied in vitro by nodal cuttings for rapid increase in number of plantlets. Thereafter, they are planted into soil or other substrates in glasshouses, either for production of transplants that are subsequently transplanted to screenhouses or the field, or for direct production of minitubers (e.g. Struik and Wiersema 1999; Tadesse et al. 2001a, b; Huarte 2005; Pruski 2007; van der Veeken and Lommen 2009; Lommen 2015; Dimante et al. 2022).

In potato, the medium composition and the light and temperature conditions employed during the last in vitro phase before planting usually do not differ from those during stock multiplication. Potato in vitro plants readily produce roots under standard multiplication conditions (e.g. Hussey and Stacey 1981) and the survival rate after transfer to the glasshouse is high. Early studies on in vitro conditions and medium composition practices in vitro and ex vitr rates in vitro (e.g. Hussey and Stacey 1981; Caligary and Powell 1989; Charles et al. 1992). However, the status of the potato plantlets at the end of the in vitro phase is also important for further performance (Kozai et al. 1988; Hagman 1990; Tadesse et al. 2000). After-effects of the photoperiod and temperature treatments employed during the in vitro phase were shown to exist on the vegetative development after planting and the later number of tubers (Seabrook et al. 1995; Tadesse et al. 2001c; Milinkovic et al. 2012). The mechanisms through which in vitro conditions affect further performance are largely unknown. Tadesse et al. (2000, 2001d) showed that a larger above-ground leaf area at planting was positively associated with a larger leaf area c. 2 weeks later. The early performance after planting is especially important when in vitro plants are used for transplant production because it affects the state of the plantlets at the beginning of the next (field) production phase and/or the time needed for the transplant production phase in the glasshouse.

In the present paper, varying the age of in vitro plantlets when planted into soil (without varying other factors in vitro) was chosen to be investigated; this would allow comparing plantlets with different stages of development and mass without concomitantly introducing major additional physiological or morphological/anatomical changes. In addition, age is a characteristic that could be adjusted easily in practical production to optimise production systems and was shown to have an effect on later tuber number (Milinkovic et al. 2012).

The objectives of this study were (1) to determine if and how the age of the in vitro plants at the moment of planting affects the early vegetative growth after planting, (2) to assess if these early differences will persist and are found across cultivars, and (3) to describe early below-ground differences that potentially may affect later tuber production.

Materials and Methods

Four experiments (Table 1) were carried out in which the duration of the last in vitro phase before planting to soil ('age') was varied. Cultivars used were Gloria (very early maturing; Exps 3–4), Bintje (mid-early maturing; Exps 1–4) and Elkana (late maturing; Exp. 4). An overview of the experimental set-ups and designs of the four experiments is supplied in Table 1, an overview of the specific

cultural practices in vitro and ex vitro is supplied in Table 2. General cultural practices in vitro and ex vitro are described later.

In *Exp. 1*, the interaction between age of in vitro plantlets at planting and the planting method was investigated. The nodes (explants) to create the plantlets were all cut on the same day from 20- and 25-day-old stock plantlets of cv. Bintje. After 10, 20, 30, and 40 days of in vitro growth, the plantlets were planted into potting soil. Thus, plantlets from different age treatments were planted on different dates in this experiment. Two planting methods were used, (1) keeping the part above the soil 'similar' or (2) keeping the part below the soil 'similar'. In (1) the upper four visible leaves were kept above the soil while burying the remaining plant parts (other stem nodes/leaves, original explant and roots; increasing in size within increase in age) in the soil; in (2) the two lowest nodes/leaves of the new shoot growing from the original explant were planted in the soil (plus explant and roots) while keeping the upper part of the in vitro plantlet (increasing in size with increase in age) above the soil. Plants were analysed at 0 and 10 days after planting (DAP). The experimental design was completely randomised in the in vitro phase and within an age treatment in the ex vitro phase, with 18 replicates per treatment.

In *Exp. 2*, it was investigated if the effects of age of in vitro plantlets found 10 days after planting would remain visible also for a longer time period. The performance of 15-, 25-, 35- and 45-day-old in vitro plantlets of cv. Bintje was compared at 10, 20 and 30 DAP. Planting to soil of all age classes of in vitro plantlets took place on the same day, whereas the explant nodes for these plantlets were cut 45, 35, 25 and 15 days before the planting date from the same stock of plants (which increased in age from 22–52 days the later the cutting). The experimental design was completely randomised in the ex vitro phase, with 25 replicates per treatment.

In *Exp. 3*, effects of age and soil cover at planting were investigated in two cultivars. The performance of 14- and 28-day-old in vitro plantlets of cvs Gloria and Bintje was compared at 1 and 13 DAP and the relation between soil cover at 1 and 13 DAP was assessed for individual plants. In Exp. 3, the timing of the multiplication of the plantlets was further refined to avoid possible entanglement of the age with the status of the stock plant from which the explant nodes were cut. The timing of multiplication now started several months in advance so that at the date of planting plantlets were available of 14 and 28 days old, which all originated from explant nodes cut from stock plants that were 49 days old. Experiment 3 had a randomised complete block design with plantlet age and cultivar as factors and with 35 replicated blocks.

In *Exp. 4*, the performance of 14-, 28-, 42- and 56-day-old in vitro plantlets of cvs Gloria, Bintje and Elkana was assessed at 0 and 14 DAP. The timing of the multiplication of the plantlets started several months in advance so that at the date of planting plantlets were available of 14, 28, 42 and 56 days, that were all cut from stock plantlets of 35 days old. Experiment 4 had a split-plot design with 24 blocks and cultivar as main factor and age as subfactor.

Culture of Plantlets In Vitro

Stock plants were multiplied using single-node cuttings. Media and conditions were the same during routine multiplication and the last in vitro phase when the

Table 1 Experimental factors and levels and experimental design in the four experiments

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Cultivar(s)	Bintje	Bintje	Gloria, Bintje	Gloria, Bintje, Elkana
Microplant age at planting	10, 20, 30, 40 days	15, 25, 35, 45 days	14, 28 days	14, 28, 42, 56 days
Growth duration ex vitro	0, 10 DAP	10, 20, 30 DAP	0/1, 13 DAP	0, 14 DAP
Planting method(s)	4 leaves above soil, 2 nodes below soil level	4 leaves above soil	4 leaves above soil	4 leaves above soil
Experimental factors	Age, planting method	Age, duration ex vitro growth	Age, cultivar (variability between plants)	Age, cultivar
Experimental design	CRD	CRD	RCBD	Split-plot
Replicate/block number	18	25	30	24

DAP days after planting

CRD completely randomised design

RCBD randomised complete block design

Table 2 Plant preparation, and experimental units, conditions and cultural practices in the in vitro and ex vitro phases of the four experiments

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Plant preparation	All in vitro plantlets were derived from 20–25-day-old mother plants, and were planted at intervals of 10 days	All in vitro plantlets were planted on one day but were cut on varying dates from one stock of mother plants, 22–52 days old at the respective dates of cutting	All in vitro plantlet age classes were planted on one day and were derived all from 49-day-old mother plants	All in vitro plantlet age classes were planted on one day and were derived all from 35-day-old mother plants
In vitro phase				
Experimental unit	Tube with one plant	Tube with one plant	Tube with one plant	Tube with one plant
Tube size ($h \times ø$)	20×2 cm	15×2 cm	20×2 cm	20×2 cm
Medium quantity (ml/tube)	9	9.5	9	9
Light conditions TL-33	16 h, 2.9 W m^{-2}	16 h, 4.7 W m^{-2}	16 h, 4 W m^{-2}	16 h, 4 W m^{-2}
Temperature	23 °C	23 °C	23 °C	23 °C
Ex vitro phase				
Experimental unit	Pot with one plant	Pot with one plant	Pot with one plant	Pot with one plant
Pot size	13×13×13 cm	10 cm Ø, 13 cm *h*	13×13×13 cm	13×13×13 cm
Fertilisation	No (short duration)	Twice per week	No (short duration)	No (short duration)
Light conditions (1:1 SON-T and HPI)	16 h, 100 W m^{-2}	16 h, 100 W m^{-2}	16 h, 170 W m^{-2}	16 h, 100 or 170 W m^{-2}, depending on block
Temperature	c. 23/23 °C	c. 20/15 °C	c. 20/12 °C	c. 20/12 °C
Relative humidity (%)	80	n.a	70	70

DAP days after planting

n.a. data not available

in vitro plantlets were produced for planting. Plantlets were cultured in glass tubes of 2.0 cm ∅ × 15–20 cm *h* with 9–9.5 ml medium containing M&S mineral salts, sucrose 25.0 g l^{-1}, agar 8.0 g l^{-1}, alar-64 (daminozide) 13.3 mg l^{-1}, glycine 2.0 mg l^{-1}, myo-inositol 100.0 mg l^{-1}, nicotinic acid 0.5 mg l^{-1}, pyridoxine HCl 0.5 mg l^{-1} and thiamine HCl 0.1 mg l^{-1}, at 23 °C, 16 h of light and a light intensity of 2.9–4.7 W m^{-2} supplied by Philips TL-84 fluorescent tubes. Details per experiment are listed in Table 2. One explant, consisting of a nodal cutting including stem part, bud and leaf, was cultured per tube. Tubes were closed with a polycarbonate cap and sealed with household plastic film.

Plant Culture Ex Vitro

Rooted in vitro plantlets were planted into potting soil in black plastic pots (one plant per pot) leaving four visible leaves above soil in Exps. 2, 3 and 4 and according to treatment in Exp.1. Pot sizes are detailed in Table 2.

Plants were grown in walk-in growth chambers. Details on conditions and cultural practices are summarised in Table 2. Radiation was supplied by a 1:1 ratio of SON-T and HPI-T lamps (Philips). The photo-/thermo-period was 16 h. Plants were fertilised twice a week in Exp. 2 starting 4 days after planting with 50 ml per plant of a low-dosed complete nutrient solution [0.472 g l^{-1} $MgSO_4.7H_2O$, 0.135 g l^{-1} KH_2PO_4, 0.140 g l^{-1} K_2SO_4, 0.034 g l^{-1} H_2SO_4(0.7 M), 0.80 g l^{-1} $Ca(NO_3)_2.4H_2O$, 0.446 g l^{-1} KNO_3, 0.035 g l^{-1} FeEDTA, 0.002 g l^{-1} $MnSO_4.1H_2O$, 0.003 g l^{-1} H_3BO_3, 0.5 mg l^{-1} $ZnSO_4.7H_2O$, 0.1 mg l^{-1} $Na_2MoO_4.2H_2O$, 0.1 mg l^{-1} $CuSO_4.5H_2O$; pH 6.0]. The duration of the other experiments was short and no nutrient solution was applied.

Observations

Length, total node number and below-ground node number of the main stem were recorded in all experiments and fresh and dry weights of different plant parts were assessed on the dates of analysis. The node number of the stem included the number of leaves visible in the apex. The dry weight of leaves of 10- and 20-day-old in vitro plantlets in Exp. 1 was too low for reliable assessment on a per plant basis and was estimated to be 10% of the fresh weight. The dry weights of the individual plant parts were combined to derive the total dry weight in Exps. 1, 3 and 4. In Exp. 2, no root and below-ground stem weights were recorded during ex vitro growth and consequently no total dry weight data were available. Leaf area and ground cover were determined by a grid method or by image analysis.

Statistical Analysis

Genstat release 17.1 (VSN International Ltd., 2014) and earlier versions were used for analysis of variance and regression analysis. Differences between treatments within significant main effects or interactions were analysed by LSD tests at $\alpha = 0.05$

(Protected Fisher's LSD test). Differences in leaf area and shoot weight in time (Exp. 2) were analysed after ln-transformation of the data.

To evaluate effects of in vitro plantlet age on fractions of plants with stolons or tubers, the numbers of plants with and without these characteristics per age treatment were subjected to chi-square analysis within a time of harvest (Exp. 2) or cultivar (Exp. 4) in Microsoft Excel (2010) to test for homogeneity of the distributions.

To evaluate the effect of age on number of stolons for those plants that did produce stolons, one-way ANOVA was performed for plants within a time of harvest (Exp. 2) or cultivar (Exp. 4) to deal with the unequal plant numbers per age class.

Results

Exp. 1: How Does the Planting Method Affect the Performance of In Vitro Plantlets of Different Age?

Differences at Planting

As expected, older in vitro plantlets were longer than younger in vitro plantlets at the moment of planting, and had more main stem nodes, a higher total dry weight (Table 3) and also had a larger total green leaf area and higher shoot and root weights (data not shown).

When planting, an important choice is which part of the stem to plant into the soil and which part to keep above soil. At least one of these parts will vary due to the increase in length of the plantlets with increase in age. Interactions between planting method and age of the plantlets were found for almost all characteristics recorded at planting (Table 3). The number of below-ground stem nodes was exactly 2 for all age classes in the planting treatment 'two nodes below soil', whereas this number of below-ground stem nodes increased from 0.2 to 8.1 with increase in in vitro plantlet age in the planting treatment 'four leaves above soil'. Conversely, the number of leaves that remained above soil at planting in the treatment 'two nodes below soil' increased with increase in in vitro plantlet age from 1.5 to 8.4 (Table 3). At the moment of planting, not all 10-day-old in vitro plantlets already had four leaves; therefore, on average only 3.2 leaves could be kept above soil in the 'four leaves above soil' treatment for 10-day-old plantlets (Table 3); in older plants of this treatment, exactly four leaves were kept above soil.

Logically, the leaf area kept above soil at planting increased with increase in plantlet age when planted with 'two nodes below soil' (Table 3); however, the above-ground leaf area also increased with increase in plantlet age when planting with 'four leaves above soil', even when exactly four leaves were kept above soil. This implies that a change in the area of the apical leaves of the in vitro plantlets occurred with increase in plantlet age. Nevertheless, the differences between the two planting methods in leaf area kept above soil increased with increase in plantlet age.

Table 3 Effects of planting method (4 leaves above ground, 2 nodes/leaves below ground) and in vitro plantlets age (10, 20, 30 or 40 days) at planting on plant characteristics at planting (0 days after planting (DAP)) and changes thereafter (0–10 DAP, 10 DAP). Data from Exp. 1, cv. Bintje. Data at 0 DAP were either obtained non-destructively from the same plantlets harvested at 10 DAP (stem length, node number) or from a separate set of plants harvested destructively (other data)

Age of in vitro plantlets at planting (Age) and planting method (Meth)	Total stem length at planting (mm)	Number of main stem nodes/leaves at planting (#/plant)			Leaf area above ground (mm^2/plant)			Total dry weight (mg/plant)		
		Total	Planted below soil	Kept above soil	At planting	Increase after planting	At harvest	At planting	Increase after planting	At harvest
Time (interval)	0 DAP	0 DAP	0 DAP	0 DAP	0 DAP	0–10 DAP	10 DAP	0 DAP	0–10 DAP	10 DAP
10-day-old in vitro plantlets										
4 leaves above soil	15.0 a	3.3 a	0.2 a	3.2 b	4.1 a	449 a	453 a	1.19 a	18.5 a	19.7 a
2 nodes below soil	16.7 a	3.5 a	2.0 a	1.5 a	2.6 a	444 a	447 a		16.6 a	17.8 a
20-day-old in vitro plantlets										
4 leaves above soil	25.1 b	7.6 b	3.6 c	4.0 c	17.2 ab	1550 c	1567 cd	1.98 a	63.7 c	65.7 c
2 nodes below soil	31.2 b	6.7 b	2.0 b	4.7 d	20.6 ab	1535 c	1557 bcd		65.8 c	67.8 c
30-day-old in vitro plantlets										
4 leaves above soil	36.4 c	9.7 c	5.7 d	4.0 c	30.3 bc	1110 b	1141 b	3.59 b	54.1 bc	57.7 bc
2 nodes below soil	38.1 c	9.3 c	2.0 b	7.3 e	43.5 c	1727 c	1770 d		85.4 d	89.0 d
40-day-old in vitro plantlets										
4 leaves above soil	45.1 c	12.1 d	8.1 e	4.0 c	46.7 c	1342 bc	1389 bcd	5.94 c	47.8 bc	53.8 bc
2 nodes below soil	38.2 c	10.4 d	2.0 b	8.4 f	104.3 d	1060 b	1173 bc		40.1 b	46.1 b
Significances[1]										
P_{Age}	***	***	***	***	***	***	***	***	***	***

Table 3 (continued)

Age of in vitro plantlets at planting (Age) and planting method (Meth)	Total stem length at planting (mm)	Number of main stem nodes/leaves at planting (#/plant)			Leaf area above ground (mm^2/plant)			Total dry weight (mg/plant)		
		Total	Planted below soil	Kept above soil	At planting	Increase after planting	At harvest	At planting	Increase after planting	At harvest
Time (interval)	0 DAP	0 DAP	0 DAP	0 DAP	0 DAP	0–10 DAP	10 DAP	0 DAP	0–10 DAP	10 DAP
P_{Meth}	ns	ns	***	***	***	ns	ns		ns	ns
$P_{Age \times Meth}$	ns	ns	***	***	***	*	*		*	*

DAP Days after planting

Similar *letters* indicate that means do not differ significantly according to Fishers protected LSD test (α=0.05). When interaction was not significant, *letters* refer to differences between age classes across both planting methods

[1]*** Significant at $P < 0.001$, ** Significant at $0.001 \leq P < 0.01$, * Significant at $0.01 \leq P < 0.05$, *ns* Not significant, $P \geq 0.05$

Performance After Planting

Effects of Plantlet Age For both planting methods, the performance after planting of the 10-day-old in vitro plantlets was very poor, with rates of increase in leaf area and dry weight in the first 10 days after planting being less than half of those in plants from older (20- to 40-day-old) in vitro plantlets (Table 3).

Among the older in vitro plantlets (20–40 days old), there were some significant differences in performance after planting but no consistent trends due to age. When planting with four leaves above the soil, the rate of increase in dry weight after planting (0–10 DAP) was not significantly different between age classes, whereas the rate of increase in leaf area was larger for 20- than for 30-day-old plantlets, with 40-day-old plantlets performing intermediate. When planting with two nodes below soil, the 20-day-old plantlets performed better than the 40-day-old plantlets in dry weight and leaf area increase after planting, whereas the 30-day-old plantlets outperformed the 20-day-old plantlets in dry weight increase, but not significantly in leaf area increase.

Effects of Planting Method The differences between planting methods in leaf area growth and total dry matter increase after planting were not significant, except for 30-day-old plantlets, for which planting two nodes below soil outperformed planting with four leaves above soil.

In subsequent experiments, too young plantlets (10 days old) were excluded, plantlets of different age were planted all with four visible leaves above soil, and plantlets of different age were planted simultaneously to ex-vitro conditions. The latter necessitated to cut different-age in vitro plantlets to be compared for growth after planting, at varying days before planting.

Exp. 2. Are Effects of the In Vitro Plantlet Age on Early Performance Persistent?

Above-Ground Development After Planting

Ten days after planting, plants grown from older in vitro plantlets (cv. Bintje) had higher leaf areas and higher above-ground shoot dry weights, with differences being especially large between plants from 15-day-old in vitro plantlets and those from the older (25-, 35- and 45-day-old) in vitro plantlets (Table 4). Differences as observed 10 DAP were still visible in plants at 20 and 30 DAP, with the leaf area of plants from the oldest in vitro plantlets being 39% higher than the leaf area in plants from the youngest plants at 30 DAP, when the experiment ended. Comparable results were achieved for above-ground shoot dry weight (Table 4).

Table 4 Effects of age of in vitro plantlets (15, 25, 35 and 45 days old) at planting on leaf area and above-ground shoot dry weight, 10, 20 and 30 days after planting (DAP) in soil. Exp. 2, cv. Bintje

Days after planting (DAP)	Age of in vitro plantlets at planting (days old)	Leaf area (cm^2/plant)	Shoot dry weight above ground (mg/plant)
10 DAP	15	9.0 a	22 a
	25	15.5 b	38 b
	35	16.7 bc	41 bc
	45	17.6 c	44 c
20 DAP	15	131.5 d	454 d
	25	162.0 e	583 e
	35	186.1 ef	670 ef
	45	207.8 f	707 f
30 DAP	15	717.3 g	3037 g
	25	884.6 h	4125 h
	35	931.0 hi	4362 hi
	45	996.6 i	4661 i
Significances[1]			
P_{Age}		***	***
P_{DAP}		***	***
$P_{Age \times DAP}$		ns	ns

[1]Significances based on ANOVA of ln-transformed data. *** Significant at $P < 0.001$, *ns* not significant, $P \geq 0.05$

Similar *letters* indicate that treatments do not differ significantly according to Fishers protected LSD test ($\alpha = 0.05$). Because interaction was not significant, *letters* are based on significances of main effects

Below-Ground Development After Planting

Plants from younger in vitro plantlets had fewer main stem nodes buried below soil than plants from older in vitro plantlets (Table 5). They also were later in initiating stolons and produced fewer stolons per plant than those from older in vitro plantlets. The number of stolons initiated increased strongly with time after planting. At 30 DAP, the number of stolons per plant in plants from the older in vitro plantlets (25–45 days old) was higher than the number of main stem nodes planted below ground, whereas in plants from the youngest in vitro plantlets (15 days old) not even all plants had initiated stolons.

Exp. 3. Is the Positive Effect on Leaf Cover of a Higher In Vitro Plantlet Age Related to a Larger Initial Leaf Cover at Planting?

In line with Exp. 1, the average above-ground leaf area of the upper four leaves kept above soil at planting and the total plant dry weight at planting (assessed 1 DAP) were higher for 28-day-old in vitro plantlets than for 14-day-old plantlets, in both cultivars tested (cv. Gloria and cv. Bintje) (Table 6). In addition, growth rates after

Table 5 Effects of age of in vitro plantlets (15, 25, 35 and 45 days old) at planting on below-ground stem nodes and stolon formation, 10, 20 and 30 days after planting (DAP) in soil

Days after planting (DAP)	Age of in vitro plantlet at planting (days old)	Below-ground node number on main stem (all plants) (#/plant)	Fraction plants with stolons (#/#)	Stolon number	
				Per plant (all plants) (#/plant)	Per plant with stolons (#/plant with stolons)
10 DAP	15	2.72 a	0.00	0.00 a	n.a
	25	4.32 b	0.08	0.08 a	1.00 a[2]
	35	5.72 c	0.16	0.28 a	1.75 a
	45	7.20 e	0.16	0.32 a	2.00 a
P			ns[1]		ns[2]
20 DAP	15	n.a	0.36	0.40 a	1.11 a[2]
	25	n.a	0.96	2.16 bc	2.25 b
	35	n.a	0.92	3.32 cd	3.61 c
	45	n.a	1.00	3.46 d	3.46 c
P			***[1]		***[2]
30 DAP	15	2.00 a	0.80	1.96 b	2.45 a[2]
	25	3.80 b	1.00	6.24 e	6.24 b
	35	6.24 cd	1.00	10.44 f	10.44 c
	45	6.92 de	1.00	9.68 f	9.68 c
P			**[1]		***[2]
Significances across all factors					
P_{Age}		***		***	
P_{DAP}		ns		***	
$P_{Age \times DAP}$		**		***	

n.a. not assessable or assessable, there were no plants with stolons

Different *letters* indicate that differences between age classes are significant based on the outcome of the ANOVA and LSD test to separate the relevant means, unless stated otherwise. For fraction of plants, *P* is based on the chi-square test and no lettering is provided

*** Significant at $P < 0.001$, ** significant at $0.001 \leq P < 0.01$, * Significant at $0.01 \leq P < 0.05$, *ns* not significant, $P \geq 0.05$

[1]Based on chi-square test

[2]Based on comparisons within the same DAP with unequal plant numbers per age class

planting and the resulting leaf areas and total dry weights at 13 DAP were higher when planting older in vitro plantlets (Table 6).

For the plants analysed at 13 DAP for leaf area, additionally ground cover by the leaves was assessed at 1 and 13 DAP. Variation in ground cover between individual plants within an age class was considerable (Fig. 1). Regression analysis showed that across age classes and across cvs Gloria and Bintje, the initial ground cover after planting (1 DAP) of an individual plant accounted for 40.1% of the variance ($R^2_{adjusted}=0.401$) in the later ground cover (13 DAP) in a linear regression model and for significantly more in a quadratic model ($R^2_{adjusted}=0.451$). Adding interaction with cultivar (allowing cultivar to affect the estimates of the coefficients of both the linear and quadratic model components of the association between later and initial ground cover) improved the quadratic model significantly to $R^2_{adjusted}=0.627$ (Fig. 1). In both cultivars, the increase in ground cover at 13 DAP with increase in initial ground cover became less at higher levels of initial ground cover. Adding 'Age' as an additional explanatory factor besides initial ground cover to the regression model, did not increase the $R^2_{adjusted}$. Comparable results were obtained when other plant characteristics at 13 DAP (e.g. leaf area, above-ground shoot dry weight) were related to the initial ground cover at 1 DAP (data not shown).

Exp. 4. Do Effects of Age Persist at Extreme Differences in Age and in Different Cultivars?

Above-Ground Leaf Area and Total Dry Weight

At planting, the total leaf area of the upper four leaves kept above ground increased with increase in age of the in vitro plantlets planted, with maximum values for c. 42-day-old plantlets in cvs Gloria and Elkana and 56-day-old plantlets in cv. Bintje (Table 7). Plantlet dry weight at planting increased over the full range of age classes in all three cultivars (Table 7).

Maximum leaf area increases after planting and maximum leaf areas at 14 DAP were realised in cv. Gloria by 42-day-old plantlets and values were clearly lower in this cultivar for 56-day-old plantlets; in the other cultivars, these variables increased with in vitro plantlet age across the full range, although the maximum values for the 56-day-old plantlets did not differ significantly from those of 42-day-old plantlets (Table 7). Similar effects were found for the increase in plant dry weight after planting and the final dry weight per plant at 14 DAP (Table 7).

Below-Ground Development

In all cultivars, plants from older in vitro plantlets had more below-ground main stem nodes than plants from younger in vitro plantlets, showed a higher fraction of plants that had initiated stolons at 14 DAP and had initiated more stolons per plant, resulting also in more positions on stolons where tubers potentially could be formed (Table 8).

Table 6 Effects of age of in vitro plantlets (14 or 28 days old) at planting on leaf area, plant dry weight and stolon formation of different cultivars (Gloria, Bintje), 1–13 days after planting (DAP) into soil

Cultivar (CV)	Age of in vitro plantlets at planting (days old)	Leaf area above ground (mm^2/plant)			Ground cover (mm^2/plant)			Total dry weight (mg/plant)		
		At planting	Increase after planting	At harvest	At planting	Increase after planting	At harvest	At planting	Increase after planting	At harvest
		1 DAP	1–13 DAP	13 DAP	1 DAP	1–13 DAP	13 DAP	1 DAP	1–13 DAP	13 DAP
Cv. Gloria	14	42.8 a	1445 b	1488 b	26.7 a	1302 b	1329 b	6.3 b	85.3 b	91.6 b
	28	75.2 b	2097 d	2172 d	36.3 c	1751 d	1787 d	11.8 d	115.4 d	127.2 d
Cv. Bintje	14	44.3 a	1042 a	1087 a	32.2 b	925 a	958 a	4.1 a	61.0 a	65.1 a
	28	61.4 b	1899 c	1960 c	40.0 d	1469 c	1509 c	7.5 c	101.5 c	109.0 c
Significances										
P_{Age}		***	***	***	***	***	***	***	***	***
P_{CV}		ns	*	*	**	**	**	***	*	**
$P_{CV \times Age}$		ns	ns	ns	ns	ns	ns	**	ns	ns

DAP Days after planting

*** Significant at $P < 0.001$, ** Significant at $0.001 \leq P < 0.01$, * Significant at $0.01 \leq P < 0.05$, *ns* not significant, $P \geq 0.05$

Similar *letters* indicate that means do not differ significantly according to Fishers protected LSD test ($\alpha = 0.05$). When interaction was not significant, *letters* are based on main effects of cultivar and age

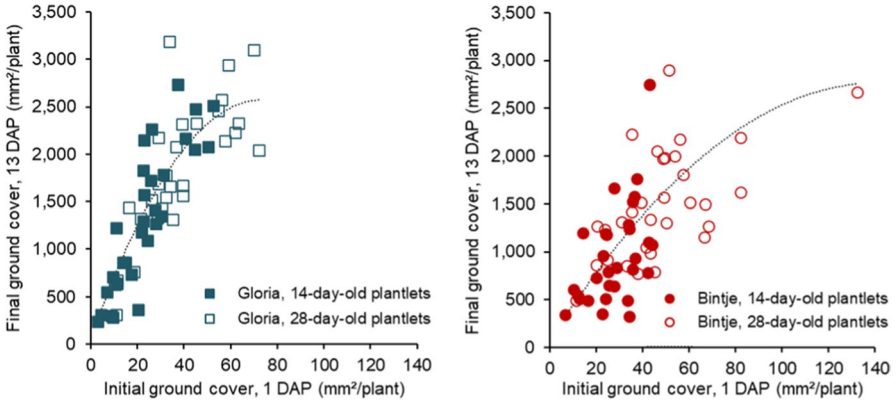

Fig. 1 Association between initial ground by the upper four leaves after planting (1 DAP) and the ground cover by the leaves at 13 DAP, of individual plants of cultivars Gloria and Bintje derived from 14- and 28-day-old in vitro plantlets, Exp. 3. Dotted lines show the cultivar-specific association according to the second-order polynomial regression model across cultivars and plantlet ages that accounts for 62.7% of the variance, as explained in the text

Tuber initiation was observed in some cases; a low fraction of plants in the oldest age classes (42 and especially 56 days old) of the very early cultivar Gloria showed already tuber initiation at 14 DAP and a single plant from the oldest age class of the mid-early cultivar Bintje (Table 8).

Discussion

Early Vegetative Development After Planting Is Poor for Young In Vitro Plantlets and Maximum for 42–56-Day-Old Plantlets

Across experiments, in vitro plantlets had ages at planting between 10 and 56 days and their early development was measured 10–14 days after planting into the soil. Fourteen days is regarded suitable period for production of transplants from in vitro-derived plantlets that will be transplanted to the field (Lommen 2015). In the present experiments, where plantlets were raised in growth chambers, well-performing plantlets had achieved leaf areas around or above 30 cm^2 at 14 DAP for (Table 7).

Leaf areas produced 10–14 days after planting were smallest—and other performance characteristics poorest—when the very young, i.e. the 10-day-old, in vitro plantlets were planted (Exp. 1, Table 3). These young plantlets even could not all be planted with four visible leaves above the soil because the newly grown shoots (grown in vitro in 10 days from the bud in the leaf axil of the original explant leaf) on average had only 3.3 visible leaves (Exp. 1, Table 3). Therefore, 10-day-old plantlets were not included in later experiments. Performance of 14–15-day-old in vitro plantlets was considerably better, but also still suboptimum because in

Table 7 Effects of the age of in vitro plantlets at planting on above-ground leaf area and total dry weight of different cultivars in Exp. 4, 0–14 days after planting (DAP) into soil

		Leaf area above ground (mm^2 per plant)			Total dry weight (TDW) (mg per plant)		
Cultivar (CV)	In vitro plantlet age (Age) at planting (days old)	At planting	Increase after planting	At 14 days after planting	At planting	Increase after planting	At 14 days after planting
Time (interval)		0 DAP	0–14 DAP	14 DAP	0 DAP	0–14 DAP	14 DAP
Cv. Gloria	14	44.5 ab	1772 a	1817 a	4.7 b	76.8 a	81.6 a
	28	51.1 abc	3380 f	3431 f	9.7 e	143.8 d	153.5 d
	42	62.2 cd	3904 g	3966 g	16.8 h	173.8 e	190.6 e
	56	54.4 bc	3241 def	3296 def	20.9 i	130.0 cd	150.9 cd
Cv. Bintje	14	40.4 a	1752 a	1793 a	3.4 a	78.9 a	82.3 a
	28	69.5 d	2611 bc	2680 bc	6.8 d	105.1 bc	111.9 b
	42	74.4 de	3054 cdef	3128 cdef	10.4 e	121.2 cd	131.5 bcd
	56	82.7 e	3313 ef	3396 ef	14.9 g	125.0 cd	139.9 cd
Cv. Elkana	14	43.5 ab	1834 a	1877 a	3.3 a	81.1 ab	84.4 a
	28	56.2 bc	2449 b	2505 b	5.7 c	106.6 c	112.3 b
	42	80.8 de	2750 bcd	2831 bcd	9.8 e	115.8 c	125.7 bc
	56	60.4 cd	2784 bcde	2844 bcde	11.1 f	119.3 cd	130.4 bcd
P_{Age}		***	***	***	***	***	***
P_{CV}		***	**	**	***	**	**
$P_{Age \times CV}$		**	**	**	***	**	***

*** Significant at $P < 0.001$, ** Significant at $0.001 \leq P < 0.01$, * Significant at $0.01 \leq P < 0.05$, *ns* Not significant, $P \geq 0.05$

Table 8 Effects of the age of in vitro plantlets (14, 28, 42 and 56 days) at planting on stolon and tuber initiation and number of below-ground main stem nodes per plant of different cultivars (Gloria, Bintje, Elkana) in Exp. 4, 14 days after planting (DAP) into soil

Cultivar (CV)	In vitro plantlets age (Age) at planting (days old)	Below-ground node number on main stem (all plants) (#/plant)	Fraction plants with stolons (#/#)	Stolon number		Potential tuber positions on stolons (all plants) (#/plant)	Fraction plants with tubers (#/#)
				Per plant (all plants) (#/plant)	Per plant with stolons (#/plant with stolons)		
Cv. Gloria	14	1.42 a	0.08	0.08 a[3]	1.00 a[2]	0.08 a[3]	0.00
	28	2.54 b	0.96	1.79 bc	1.87 a	2.96 b	0.00
	42	4.17 d	1.00	2.33 cd	2.33 a	4.25 c	0.08
	56	6.96 g	1.00	3.69 e	3.70 b	6.31 d	0.20
P			***[1]		***[2]		*[1]
Cv. Bintje	14	1.17 a	0.09	0.25 a[3]	1.20 a[2]	0.25 a	0.00
	28	3.13 c	0.79	1.46 b	1.84 ab	1.79 b	0.00
	42	5.54 e	0.92	2.58 d	2.82 b	3.71 c	0.00
	56	6.92 g	1.00	4.79 f	4.79 c	6.25 d	0.04
P			***[1]		***[2]		ns[1]
Cv. Elkana	14	1.04 a	0.21	0.21 a[3]	1.00 ab[2]	0.21 a	0.00
	28	3.33 c	0.88	1.46 b	1.67 a	2.04 b	0.00
	42	4.67 d	1.00	2.38 cd	2.38 b	3.67 c	0.00
	56	6.33 f	1.00	3.17 e	3.17 c	4.89 d	0.00
P			***[1]		***[2]		
Significances across all factors							
P_{Age}		***		***		***	
P_{CV}		*		*		*	
$P_{Age \times CV}$		***		**		ns	

Different *letters* indicate that differences are significant based on the outcome of the anova and LSD-test to separate the relevant means, unless stated otherwise. For fraction of plants, P is based on the Chi-square test and no lettering is provided

*** Significant at $P < 0.001$, ** Significant at $0.001 \leq P < 0.01$, * Significant at $0.01 \leq P < 0.05$, *ns* Not significant, $P \geq 0.05$

[1]Based on Chi-square test

[2]Based on comparisons within the same cultivar with unequal plant numbers per age class

[3]*Letters* reflect the effect of age based on the main effect of age being significant

Exps 2–4 they consistently led to plants with lower leaf areas than when planting in vitro plantlets of 25–28 days old (Tables 4, 6, 7). The leaf area produced at 10–14 DAP even increased further when the in vitro plantlets had been older at planting up to c. 42- or 56- day-old (Table 7), but this increase with increase in age gradually became smaller, especially when plantlets had been older than c. 28 days at planting (Tables 4, 7). In Exp. 4, the leaf area produced at 14 days after planting increased with age of the in vitro plantlets used up to an age of c. 42 days and thereafter declined in cv. Gloria, levelled off at c. 42 days in cv. Elkana and tended to increase further with increase in age up to 56 days in cv. Bintje (Table 7).

Effects of plantlet age on early leaf area remained visible until at least 30 DAP, the last day of measuring in Exp. 2 (Table 4).

During the experiments, the timing of the plantlet production and planting was gradually refined to exclude possible risk of confounding effects of the age of in vitro plantlets at planting with effects of unintended differences in conditions and day-to-day management after planting when different-age plantlets were planted on different dates (Exp. 1), or with possible effects resulting from a different age of the mother plants from which the explant nodes were cut at different time intervals before planting when planting on the same date (Exp. 2). Therefore, the timing of the last multiplication steps in vitro was advanced, so that at the date of planting, plantlets were available of the desired age, that all originated from explants cut from stock plants of the same age (Exps. 3 and 4). These methods will have limited the risk of confounding factors interfering with age effects.

It is not clear how other authors dealt with these timing effects, because in the papers published thus far on age of in vitro plantlets (Hassanpanah and Khodadadi 2009; Milinkovic et al. 2012; both focussing on tuber production after planting in a glasshouse) no details were provided on the timing of planting.

Better Early Performance Was Related to a Higher Above-ground Leaf Area After Planting and Higher Growth Rates Thereafter

The larger leaf areas at 10–14 DAP of plants grown from older in vitro plantlets seemed to be caused by (1) a larger initial above-ground leaf area of the older in vitro plants at the moment of planting (Tables 3, 6, 7) and (2) a higher absolute rate of increase in leaf area thereafter (Tables 3, 4, 6, 7).

The *larger leaf area at planting* for older in vitro plants (Exps. 1, 3 and 4) was found despite plantlets of all ages were planted with four visible leaves above the soil surface. The leaf area of these upper four leaves kept above soil at planting increased with age of the in vitro plantlets up to an age of at least 42 days (Tables 3, 4, 7). This shows that the phenology of the apical shoot part (upper four leaves) of the plantlets changes during growth in vitro, with the earlier formed leaves (forming the top leaves in younger plants) increasing less rapidly in size after emerging from the apex than the later formed leaves. It is likely that the phenology of the upper leaves will not just be influenced by age and cultivar, but also by changing the abiotic conditions in the in vitro environment. There is little quantitative information on this available for specifically the four upper apical leaves of potato but addition of

daminozide to the medium, cooler temperatures in vitro and reduction of the N concentration in the medium can increase the total leaf area of the leaves on the upper half of the stem of in vitro plantlets (Tadesse et al. 2000).

The *higher rate of leaf area increase* between planting and 10/14 DAP when planting older in vitro plantlets may be determined to a large extent by the initially higher leaf area after planting, because across the plant ages studied in the individual plants in Exp. 3 (14- and 28-day-old plants) ground cover at 13 DAP was shown to be highly associated with the ground cover at 1 DAP (Fig. 1). Also in 30-day old plantlets in Exp. 1 (Table 3) shallower planting (keeping slightly more leaves above soil) resulted in a higher leaf area kept above soil at planting (43.5 vs. 30.3 mm^2) and a higher rate of increase thereafter. The importance of the initial above-ground leaf area after planting for later leaf area confirms earlier findings by Tadesse et al. (2000, 2001d). It remains to be solved if this is through a higher radiation interception resulting in more production or because of a different boost in leaf area increase because of a different predisposition/potential of the apical leaves and the leaves that still need to appear from the apex after planting. In vitro plantlets usually show a boost in production after planting to ex vitro conditions (Tadesse et al. 2001e).

Yet, the association between the initial above ground leaf area and the later leaf area may level off at higher values of initial leaf area or may even become negative when (many) more than four leaves are kept above soil when planted. The levelling off is suggested by Fig. 1 for ground cover of individual plants (all planted with the upper four leaves above soil) and the negative influence by the poor performance of the oldest shallow-planted plants in Exp. 1 (40 days old; Table 3), where leaving extra (in total 8.4) leaves above soil at planting, with a total leaf area of 104.3 mm, performed less well than planting 4 leaves above soil with a total leaf area of 46.7 mm^2. When keeping too many leaves and leaf area above soil at planting, the roots may not be sufficiently active to support an increased transpiration. In addition, planting shallow (as in Exp. 1) was very contra-intuitive for older plantlets, because of the longer, vulnerable (thin) stem part kept above soil leading to less robust plants than planting with 4 leaves above soil.

Positive effects of a higher age of the in vitro plantlet on the early growth after planting were also visible for other plant characteristics than leaf area, like total dry weight (Tables 3, 6, 7); values were higher because of higher values at planting and/or higher absolute growth rates thereafter (Tables 4, 6, 7). Total plant dry weight at planting increased with age of the in vitro plantlets across the full range of ages studied. The higher initial leaf area and leaf area increase (Tables 3, 4, 7) will have been an important factor steering the growth rates after planting.

Stolon and Tuber Formation Are Delayed when Planting Younger In Vitro Plantlets

Stolon and tuber initiation were clearly delayed when planting very young in vitro plantlets; this was shown in the proportion of plants with stolons or tubers and the number of stolons per plant.

Experiments 2 and 4 showed a lower *proportion of plants* with stolons at 10/14 DAP when planting younger in vitro plantlets than when planting older (Tables 5,

8), and this situation was maintained up to 30 DAP, the latest day of assessment in Exp. 2 (Table 5). Two different mechanisms might explain this. Firstly, younger in vitro plantlets are smaller and therefore had to be planted shallower. This left fewer stem nodes buried in the soil as potential sites of stolon production than after planting older plantlets (Tables 5 and 8); moreover, these few below-ground nodes were close to the soil surface and may miss the dark conditions that promote stolon development (cf. Kumar and Wareing 1972). Secondly, very young plantlets may be less advanced in the stolon formation process, still needing additional triggers to start the stolon initiation.

The on average higher *numbers of stolons* observed in plants from older in vitro plantlets were due to a higher proportion of plants having stolons—as discussed above—and a higher number of stolons per plant in the plants possessing stolons. The higher stolon numbers in plants from older in vitro plantlets may be explained at least partly by the higher number of main stem nodes buried below ground, also leading to more positions where tubers potentially could be formed (cf. Table 8). Yet, the number of stolons could be higher than the number of main stem nodes in these in vitro-derived plants (Table 5) which already indicates that more than one stolon was produced per below-ground main stem node buried below ground. So, in these types of plants, stolons will not only originate from the central axillary buds on the main stem but will also arise from the ancillary buds (small buds in the axils of 'leaf' primordia surrounding the central bud). Especially during later development, stolons may also arise from subterranean buds on branches of the main stem or on side shoots originating from stolons turning upwards to become leafy shoots; in addition, stolons may arise above ground, but close to the ground level, from nodes on the lower main stem or side shoots (observations by the author).

Also *tuber initiation* was delayed when planting younger compared to older in vitro plantlets. Tuber initiation was rarely observed in the first weeks after planting, but when observed it was in plants from the older in vitro plantlets and the earlier cultivars (Table 8). The delay in tuber initiation can be explained by plants from younger in vitro plantlets and later cultivars being less advanced in the tuber formation process, still needing additional external or internal stimuli to start tuber initiation. In addition close proximity of the subterranean buds to the active, above-ground stem and leaves may have limited early tuberisation in the plants from the younger in vitro plantlets (cf. Khan and Ewing 1983). The age effects are in line with the observations by, e.g., Hussey and Stacey (1981) that also under in vitro conditions, plantlets will eventually initiate tubers when kept in vitro for a long time.

Postamble

Commonly in vitro plantlets are planted to ex vitro conditions 2–3 weeks after cutting. The present paper suggests that this reduces the early growth and leaf area development after planting compared to planting in vitro plantlets of 25–28 days or older, and that this backlog remains visible until at least 30 days after planting. However, in the present experiments, in vitro plantlets were grown individually in tubes, also in the last phase before planting, and therefore had a relatively large quantity

of medium available per plant. It is likely that when more in vitro plantlets are cultivated together in a Petri dish or small container—a common practice during the last in vitro phase in many labs—they will earlier deplete the medium for nutrients (cf. Schum and Jansen 2012) and water and may more easily get damaged due to a narrow headspace (depending on the container), but it is not yet clear how this will affect the apical constitution and performance of plants after planting.

The better performance of older in vitro plantlets was associated with a larger leaf area of the upper four leaves kept above the soil at planting and with a higher increase after planting. The higher leaf area kept above soil was associated with differences in growth of the apical leaves during in vitro growth. It remains to be solved if the higher increase after planting is fully explained through a higher radiation interception resulting in more production, or if it is also linked to a different predisposition of the leaves separating from the apex after planting. Other factors affecting the leaf area kept above soil after planting could also be changed conditions in vitro or simply keeping more leaves above soil at planting. However, keeping more leaves above soil (Exp. 1; Table 3) will not necessarily successfully increase growth (Table 3); roots may not be sufficiently active to support an increased transpiration and plants may become less robust whereas also the importance of newly appearing leaves may be higher than that of the existing leaves for plant growth.

Obviously, 10/14 days growth ex vitro—at higher radiation levels and lower plant densities than in vitro—resulted in a higher increase in dry weight than 10/14 extra days of in vitro growth (cf. Tables 3, 4, 6, 7). This was true for all age classes, including the oldest for which the data of 10/14 days extra in vitro growth are not shown in the tables. In commercial production situations also economic and logistic considerations will play an important role in scheduling how long to grow the plantlets in vitro and ex vitro.

Early stolon formation was enhanced by planting older in vitro plantlets and this possibly may become advantageous when aiming in a later phase at high numbers of tubers per plant (cf. Milinkovic et al. 2012). The data also show that plants from older in vitro plantlets may be more advanced in the tuber formation process, especially when from early cultivars.

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Declarations

Conflict of Interest The author declares no competing interests.

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Authors and Affiliations

W. J. M. Lommen[1]

✉ W. J. M. Lommen
willemien.lommen@wur.nl

[1] Centre for Crop Systems Analysis, Wageningen University & Research, Bornsesteeg 48, 6708 PE Wageningen, Netherlands