



**Szent István University**

***In vitro* propagation of *Narcissus angustifolius* Curt.,  
a protected species in the Carpathian Mountains**

*Doctoral thesis*

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**Gödöllő**

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## 1. INTRODUCTION AND THE AIMS OF THE STUDY

Subcarpathia is a distinguished area in Ukraine, because 268 species of the Red Data Book of Ukraine (which contain 826 protected plant and fungus species) can be found here (DIDUKH, 2010). In accordance with the World Conservation Strategy, *in situ* and *ex situ* conservation of species are equally necessary.

Ukrainian white daffodil taxa were mentioned in previous and recent Red List and Red Data Book of Subcarpathia and Ukraine (SYTNIK, 1980; TAKHTAJAN, 1981; TASYENKEVYCH et al., 1982; KOMENDAR et al., 1987; KOMENDAR, 1996; KRICSFALUSY and BUDNIKOV, 1999; MALYNOVSKYI et al., 2002; SOBKA, 2002; SOBKO, 2007; ZYMAN and BULAH, 2009; Internet 1.-3.). Although, stands were diminished (some population perished) due to the soviet agriculture methods and illegal harvesting of the attractive flowers. Therefore wild daffodil populations were protected by law in formerly (during the Union of Soviet Socialist Republics) and currently.

*Narcissus angustifolius* (a protected daffodil species) was threatened by overexploitation i.e. collecting of flowers during blooming period (KOMENDAR, 1996). In order to inhibit illegal harvesting and enrich daffodil-assortment, potentiality of cultivation was proposed. Micropropagation was potentially the safest and quickest way of propagation, because some years ago a daffodil cultivar have already (and successfully) micropropagated with the use of benziladenine (BA) (JÁMBORNÉ BENCZÚR et al., 1989).

The Subcarpathian Daffodils' Valley has been protected since 1979, and this 256 ha reserve (placed in Máramaros Basin) was under by the authority of Carpathian Biosphere Reserve (UNESCO). (CHOPYK, 1970a,b). Prof. Vaszil Komendár (Uzhgorod National University, Department of Botany) has been instrumental in starting my studies and I was given an admittance from him.

### **The following aims were proposed:**

To process complete micropropagation technology of *Narcissus angustifolius*, including:

- to produce sterile cultures and to start *in vitro* propagation with combination of PB and BA,
- to achieve higher multiplication rate (during *in vitro* propagation) with the use of BA + PB,
- to increase the number of differentiated large bulblets (during *in vitro* propagation) with BAR + PB combination,
- to decrease vitrification (with an optimal multiplication rate) during shoot-induction trial,
- to ascertain the accessories of optimal rooting medium,
- to acclimatize the bulblets,
- foremost to use paclobutrazol during micropropagation of daffodil.

## 2. MATERIAL AND METHOD

During 2009-2013, micropropagation trials were carried out in the laboratory of the Department of Floriculture and Dendrology, Corvinus University of Budapest.

Bulbs (95% of them have already been dormant) were collected on 4 July, 2009. 20 bulb were prepared for sterile culture. After it, uncleaned bulbs were stored (on temperature 20-25°C) until starting.

### 2.1. Starting

#### First starting of sterile culture

Soil was washed down and withered tunic was removed from the bulbs (until the white, live scales, figure 1). Bulbs have developed new roots, so I had to prune them (with some part of basal stem).

Bulbs were sterilized (on 20 July, 2009) as the following procedure:

- At first, bulbs were washed under running tap water (supplemented with 1-2 drop of Tween-80) for 1 hour (figure 2).
- Then, bulbs were sterilized in 70% ethanol and 2‰ HgCl<sub>2</sub> for 10-10 minutes (figure 3).
- All of them were rinsed in distilled water (in sterile, laminar flow cabinet),
- At last, uncut bulbs were placed on hormone-free, incubation S-medium (figure 4), on 4°C (in order to vernalization) for 6 weeks.

As the first step of starting, bulbs were cut into 4-6 segments. After it, every explant were placed on half-strength MURASHIGE and SKOOG (MS, 1962) medium with 1 mg/l BA + 0.1 mg/l NAA (on 1 September, 2009). During incubation, sterile-like bulbs were bacterially infected, so the first 5 bulbs were perished.

Survived bulbs were repeatedly sterilized in the Gram-positive bactericide malachite (ZATYKÓ, 1992) solution, then the same (cut and uncut) bulbs were transferred to medium containing 3 g/l malachite. In spite of it, bulbs were infected again.

The cause of infection was a Gram-negative, unknown bacterium (which was proved by a bacteria-test). Thus, residual 5 bulbs were soaked in solution supplemented with 250 mg/l Cefotaxime (an efficient antibiotic against Gram-negative bacteria, HEGEDŰS, 2005) + 3 mg/l malachite. After it, bulbs (in test-tubes) were rotated for 1 week, later placed on E1C medium with 200 mg/l Cefotaxime + growth regulators (1 mg/l BA + 0.1 mg/l NAA). From these prepared explants (sliced scales), 8 specimen were proved to be sterile, and 6 of them developed shoot. Shoot-differentiation was very slow due to the antibiotic (which inhibited shoots' growth).



**Figure 1.** Cleaned bulbs



**Figure 2.** Bulbs were washed under running tap water



**Figure 3:** Bulbs were transferred from 1 %  $\text{HgCl}_2$  solution to distilled water



**Figure 4:** Uncut bulbs were placed on hormone-free (S) medium

On 15 January 2010, sterile stock (with few bulbs) was bred on E1 medium without Cefotaxime. Heterogeneous progeny was detected from subcultures, so vitrified shoots were transferred to hormone-free (S), and normal, dimidiated bulbs were placed on E1 medium. Data were evaluated on 1 July 2010 and 3 March 2011. Only averages were determinate due to the heterogeneous and few specimens.

#### **New starting (with different cytokinins) of sterile culture**

Because of the very slow and insufficient multiplication, I had to collect additional 20 bulbs from their natural habit in 2010. Half of the individuals were sterilized through the described way, and I have gained 6 healthy bulbs (which were cut and transferred to multiplication media after pre-treatments).

As a new process, some bulb was pre-treated by BA (during incubation). Furthermore, rarely used accessories as meta-topoline (MT) and paclobutrazol (PB) were added, too. After

sterilization (on 17 July 2010), I transferred uncut bulbs on S medium containing BM macroelements (JÁMBORNÉ BENCZÚR and MÁRTA, 1990) and HELLER (1953) microelements and with or without BA. After incubation, true starting (with bulb-cutting) was carried out on 13 September 2010.

Accessories were shown on table 1.

**Table 1:** Accessories of media of the 2nd starting (pre-treatment and true starting)

Medium (pre-treatment)	BA (mg/l)	Medium (true starting)	PB (mg/l)	BA (mg/l)	MT (mg/l)	IAA (mg/l)
S	-	PB1	2,5	0,5	-	0,1
S	-	PB2	2,5	1	-	0,1
E1	1	PB3	0,25	1	-	0,1
E1	1	E1	-	1	-	0,1
S	-	E1	-	1	-	0,1
S	-	MT1	-	-	1	0,1

Data were evaluated on 10 February 2010. Only averages were determinate, because I could use only 1-1 bulb to make explants (scales segments) for every combination.

## 2.2. Media of certain trials

50 ml (starting, multiplication and rooting) medium was filled into every (100 ml sized) Erlenmeyer flasks and every flasks were covered by aluminum foil before autoclaving (120 °C, 10<sup>5</sup>Pa pressure unto 30 minutes). For alimentation of carbohydrate, 30 g/l saccharose was applied and 10 g/l agar was solidified medium. The pH was adjusted to 5.5 using 1 M KOH.

### 2.2.1. Applying paclobutrazol during multiplication (2nd trial)

The aim of this trial was to gain more shoots than the earlier treatments. In order to reach it, PB (which had positive effect during the 2nd starting) was used in different concentration and combination (table 2).

**Table 2:** Accessories of media (2nd trial)

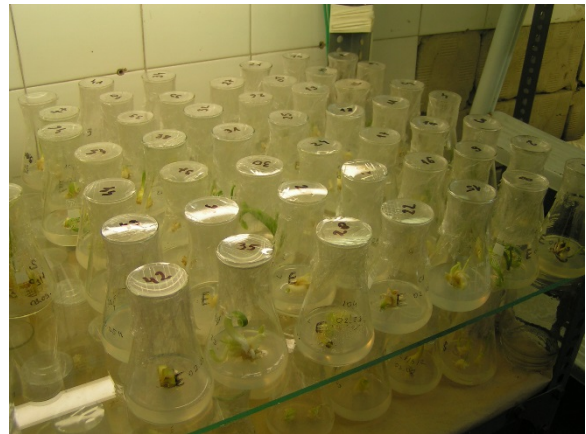
Medium	BA (mg/l)	PB (mg/l)	NAA (mg/l)
PB1	0,5	2,5	0,1
PB2	1	2,5	0,1
PB3	0,5	0,25	0,1
PB4	1	0,25	0,1
E1	1	-	0,1
E0,5	0,5	-	0,1
S	-	-	-

### 2.2.2. The after-effect of paclobutrazol on hormone-free medium (5th trial)

Based on my experiences, PB affected shoot-differentiation (but later, vitrification was detected).

The aim of this study was both to eliminate this malformation and to differentiate small bulbs with shorter period of PB-treatment (after meristem-induction was occurrence on hormone-free medium).

As shown on figure 5, sliced scales of sterile bulbs (which have differentiated during previous trials) were placed on induction medium with 1 mg/l BA + 1 mg/l PB + 0.1 mg/l NAA (1-2 inoculum per flask). Later, 3 groups were separated, and every one of them was transferred once to hormone-free ½ MS basal medium on 30 January, 10 and 23 February (there were 11-13 days between every shifting). Thus, the effect of induction period was examined. Data were evaluated 6 times (during 2012): 27 March and April, 08 and 29 May, 19 June, in fine 17-21 September.



**Figure 5:** Stock of the 5th trial (on induction medium with PB)

### 2.2.3. Media of rooting

This trial was started on 29-30 January 2013 and evaluated on March 3-5.

½ MS basal medium was used (and all accessories were shown on table 3). Daffodil-bulbs have successfully rooted on hormone-free medium (as I experienced in my previous trials) and I supposed that NAA eventuate better results. PB was also used (at the same concentration) as root-stimulator, and both agent were added to the medium in the hope that combination result more roots.

Bulbs (collected from the preceding trial) were placed on rooting media. During examination, total bulbs (i.e. bulb + roots + shoots) were measured, too (figure 6.).

Cultures were kept on 16/8 hour photoperiod and 3000 lx luminance during multiplication and rooting (in the case of every growth regulator).

**Table 3:** Accessories of media (rooting)

Medium	PB (mg/l)	NES (mg/l)
E0	0	0
GN1	0	0,1
GN2	0	0,2
GN3	0,1	0
GN4	0,2	0
GN5	0,1	0,1

### 2.3. Acclimatization

After rooting, successfully rooted bulbs were planted in plastic container (filled with 50% peat + 50% perlite) on 5 March 2013. After it, individuals acclimatized in a fitotron growth chamber unto 1.5 month with 60-80% RH (further abiotic parameters of fitotron were shown on table 4).



**Figure 6.** Measuring the weight of total bulb

**Table 4:** Abiotic parameters in the fitotrone (from 5 March 2013)

Time of day (hour)	from 6	from 9	from 11	from 17	from 20	from 21
Temperature (°C)	22	23	25	24	23	22
Luminance (lx)	4000	11500	11500	11500	4000	0

First examinations were done on 16 April 2013, and then I transferred all plants from the fitotron to the glasshouse, where stocks were kept on 18-23 °C with natural light conditions. On May 14 and June 16 further investigations were carried out and different data (for example leaf-developing, bulb viability and root-outgrowing of the bottom of containers) were evaluated as percentages.

### 2.4. Evaluation method, statistical analysis

Data were evaluated from 20-20 cultures. Small (1-9mm) and large (more than 10 mm) shoots were equally regenerated, thus I had to recorded data separately. The number and length (mm) of roots (during not only rooting but also multiplication trials), and similar features of leaves, fresh weight (g) of bulbs (in the case of rooting phase) were examined. Vitrification (ratio) was recorded in some cases.

Data recording, constructions of diagrams, tables were done by Microsoft Excel. In the case of the first 2 multiplication trial and acclimatization, software of Ropstat was used for statistical calculations (one-way analysis of variance with a confidence level  $p < 0.05$ ). In other trials of multiplication and rooting, data were evaluated by one-way analysis of variance (Tukey



HSD,  $p < 0.5$ ) with SPSS software package. Bars and letters represent significant differences in certain graphs and tables.

### 3. RESULTS

#### 3.1. Starting

##### **First starting of sterile culture**

After 1 week refrigeration, sterilized bulbs were seemed to be healthy (on 20 July 2009) in the air-conditioned light-room. Some of them (which were transferred to induction medium on 14 September 2009) bacterially infected, but after an antibiotic treatment (with the use of Cefotaxime), 6 of 8 sterile bulbs shown very slow shoot-differentiation.

From the first starting (14 weeks after sterilization by Cefotaxime) only 7 explants from 1 bulb remained healthy, so the sterilization ratio was 10%). Averagely 1 shoots was regenerated from each bulb-scale. Swollen scales (with base part of the bulb) were sliced, and segments were kept on E1 medium.

After 18 weeks variable results were observed. Some segments produced more (and small), some ones developed few but large shoots. Vitrificated shoots were also found and placed them on hormone-free S-medium. Larger shoots were cut again and transferred to medium E1.

10 months later I had got 15 sterile cultures (in different number of shoots) on S and E1 media. In the case of S, vitrificated inoculums produced averagely 5.2 shoots (with unified structure) and 18.2 roots. On E1 medium, newly differentiated shoots' number was 5.8 (with only 2.1 roots), although most of shoots needed transferring to hormone-free S medium due to the high ratio of vitrification.

##### **New starting (with different cytokinins) of sterile culture**

During this successful trial (which was started from the summer of 2010 and combined with pre-treatments), 6 group were separated and the best was PB2 medium (after incubation without hormones), where 8.7 shoots were achieved (table 5). With similar pre-treatment, PB1 medium effect 4.5 shoots/explant but significantly smaller bulblets. All (100%) shoots produced roots (also on the medium with PB2), and the average number of roots was the highest (12.5) in this case. Similar results was obtained on PB3 multiplication medium (with an incubation on medium E1), although rooting was decreased (50%).

**Table 5:** Data of regenerated shoots on different media (during 2<sup>nd</sup> starting)

Medium (pre-treatment)	Medium (true-starting)	shoot number	shoot length (mm)	shoot width	root number	rooted explants (%)
S	PB1	4.5	4.3	2.1	12.5	100
S	PB2	8.7	9.4	5.5	4.3	100
E1	PB3	3.7	7.7	6.4	2.7	50
E1	E1	2.1	3.4	2.5	1.3	33
S	E1	0.4	12.6	9.0	0	0
S	MT1	0.6	12.0	6.0	0	0

Pre-treatment with BA proved to be efficient only if true-starting medium contained only this cytokinin (in this case averagely 2.1 shoots were found). The worst combination was hormone-free medium (as pre-treatment) and E1, MT1 media (as true-starting): not more than 1 shoot (but larger bulblets without roots) were observed.

### 3.2. The effect of media (some trials of multiplication)

#### 3.2.1. The effect of paclobutrazol (2nd trial)

During the 2nd starting, PB proved to be effective, so the effect of different concentration of PB on the shoot number was examined in this trial.

As shown on table 6 (in the case of shoot number of small bulblets), PB3 (figure 11) and PB4 (figure 12) medium was significantly the best for shoot differentiation (as compared with the other media). Although PB4 result the most shoots (7.75), and the second best was PB3 (6.65), there were not significant differences between of these values. In the case of applying hormones, the fewest number of shoots (2.35) was observed on PB1 (figure 9) and E1 (figure 13) medium. PB2 (figure 10) shown similar results like E0,5. Less concentrations of hormones effected more shoots. To compare with the shoot lengths of PB1 and PB4 (5.5 and 5.58 mm), significantly the longest shoots (7.65 mm) were achieved on medium E1.

Examining large bulblets (table 7.), PB3 effect the highest number of shoots (2.5), significantly higher than E0,5 (1.1). Except control (S medium: 28.5 mm), the longest shoots (22.63 mm) were found on medium PB4, and (contrasting with control) significantly the shortest ones (16.8 mm) on E0,5.

As shown on table 8, PB4 medium effect few and short roots advantageously, because root formation is undesirable during multiplication, shoot differentiation. In the case of rooted individuals, roots must be pruned before the next transferring in order to the easier and quicker handling. The fewest roots (0.75, 0.85) with ideally short lengths (2.9 and 4.75 mm) and low percentage of rooting (7-7%) were obtained on PB1, PB2 medium, although the number of small bulblets was too low. Disadvantageously, all cultures developed roots (100%) on medium with

(or without) low concentration of hormones, but increasing of hormone level decreased root differentiation (figure 7). Except control (9.85), significantly the most roots (4.15 and 3.7) were found on medium PB3 and E1 (as compared with the results of PB1, PB2, PB4, E0,5). Roots elongated mostly on control (33.88 mm), E0,5 (16 mm) and E1 (8.68 mm) medium (and almost every cases – except E1 – significant differences were detected compared to the other treatment).

**Table 6:** Number and length of small bulblets on different media

	Small bulblets (average values)	
Medium	shoot number	shoot length (mm)
PB1	2.35 ± 1.09 a	5.50 ± 2.78 a
PB2	3.15 ± 1.04 a	6.50 ± 1.86 ab
PB3	6.65 ± 2.80 b	6.25 ± 3.55 ab
PB4	7.75 ± 4.54 b	5.58 ± 1.18 a
E1	2.35 ± 1.09 a	7.65 ± 2.03 b
E0,5	3.70 ± 0.87 a	7.18 ± 1.41 ab
S	0 c	0 c

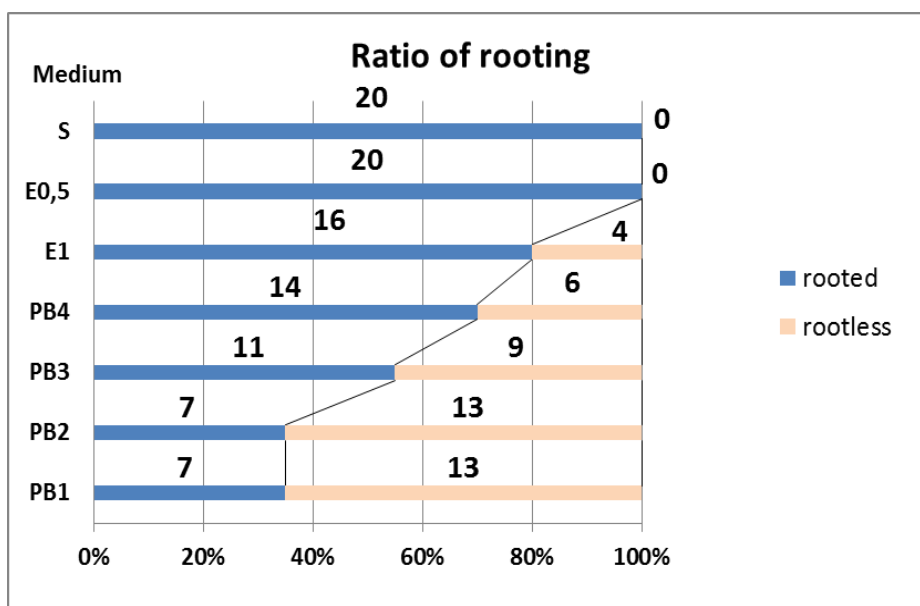
Vitrification was observed in some cultures (figure 8-10, 14), but not in the case of hormone-free (S) medium. Higher concentration of PB (PB1, PB2 and PB3) enhanced vitrification rate, especially in the case of first 2 medium (60 and 70%, figure 8-10). For surviving, some part of these cultures needed new (and hormone-free) medium.

**Table 7:** number and length of large bulblets on different media

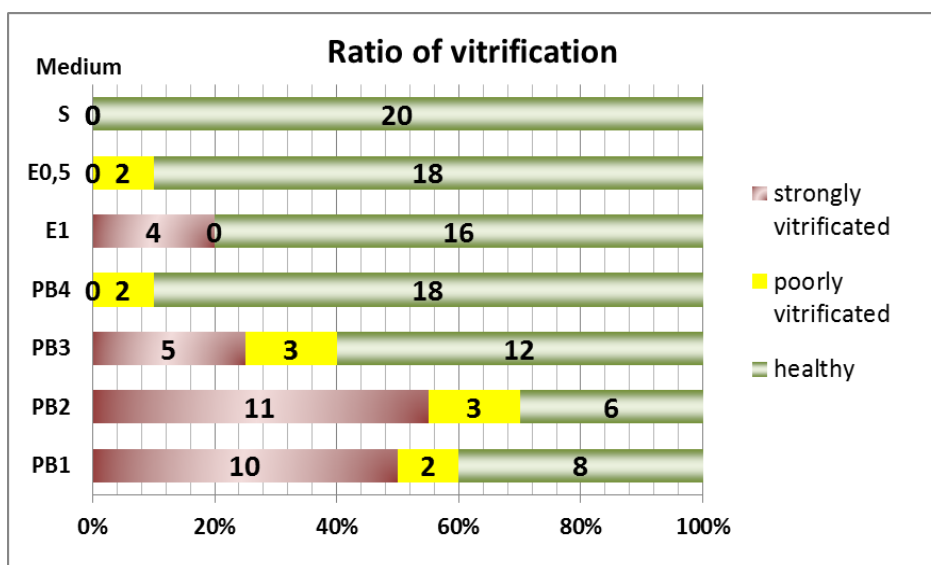
	Large bulblets (average values)	
Medium	shoot number	shoot length (mm)
PB1	1.40 ± 0.60 ab	20.23 ± 9.71 a
PB2	1.50 ± 0.76 ab	21.93 ± 6.21 a
PB3	2.50 ± 3.62 a	20.75 ± 6.96 a
PB4	1.15 ± 0.37 b	22.63 ± 6.61 ab
E1	1.45 ± 0.69 ab	20.52 ± 9.09 a
E0,5	1.10 ± 0.31 b	16.8 ± 3.07 a
S	1.50 ± 0.69 ab	28.5 ± 5.98 b

**Table 8:** The number and length of roots on different media

Medium	Roots (average values)	
	root number	root length (mm)
PB1	0.75 ± 1.12 a	2.90 ± 4.90 a
PB2	0.85 ± 1.31 a	4.75 ± 11.39 abc
PB3	4.15 ± 5.04 b	4.30 ± 5.12 abc
PB4	1.60 ± 1.54 ac	4.65 ± 3.49 abc
E1	3.70 ± 2.56 bc	8.68 ± 5.43 c
E0,5	1.30 ± 0.47 a	16.00 ± 5.03 d
S	9.85 ± 2.37 d	33.88 ± 4.55 e



**Figure 7:** Rooting percentages on different media



**Figure 8:** Vitrification (hyperhydration) on different media



**Figure 9:** Strongly vitrified large and small bulblets from medium PB1



**Figure 10:** Poorly vitrified large and small bulblets from medium PB2



**Figure 11:** One large and several small bulblets from medium PB3



**Figure 12:** Small (but visible) bulblets surround the large one (from medium PB4)



**Figure 13:** Large and small bulblets from medium E1



**Figure 14:** Poorly vitrified bulblets from medium E0,5

### 3.2.2. The after-effect of paclobutrazol on hormone-free medium (5th trial)

At the beginning (on 27 March 2012), the first group, after it (during April and May) the second one, finally (in June and September) the first stock produced the most bulblets (table 9). The

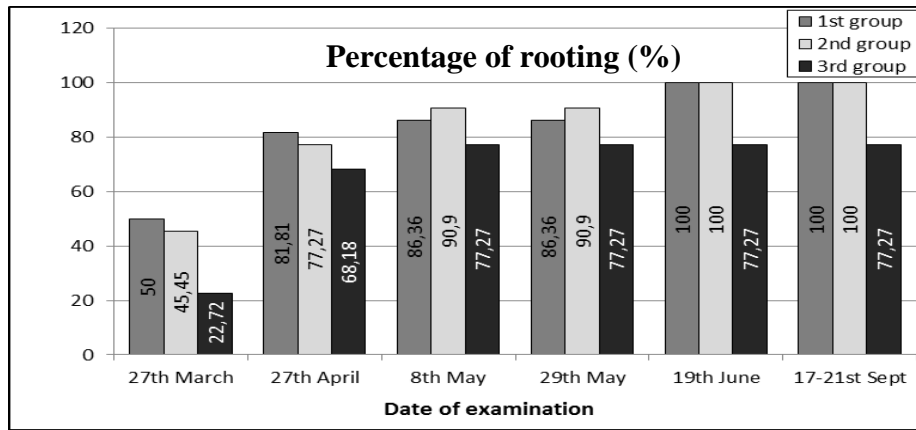
latter month, significant difference was detected between the number of bulblets of the 1<sup>st</sup> and 3<sup>rd</sup> group (4.9 and 3.68).

**Table 9:** Average number of bulblets and roots of *in vitro* *N. angustifolius* cultures

Examination time (2012)	Group	Bulblet number	Root number
27 March	1.	0.95 ± 1.21 a	1.54 ± 2.11 a
	2.	0.5 ± 0.8 a	1.09 ± 1.5 a
	3.	0.4 ± 0.66 a	0.54 ± 1.22 a
27 April	1.	1.31 ± 1.17 a	2.72 ± 2.27 a
	2.	1.18 ± 1.29 a	2.54 ± 2.08 a
	3.	1.68 ± 1.64 a	3.4 ± 3.2 a
8 May	1.	1.9 ± 1.3 a	4.59 ± 2.93 a
	2.	1.86 ± 1.39 a	4.4 ± 3.2 a
	3.	2.54 ± 2.13 a	6.54 ± 6.37 a
29 May	1.	2.4 ± 1.36 a	6.13 ± 3.72 a
	2.	2.27 ± 1.63 a	5.09 ± 3.63 a
	3.	2.95 ± 2.25 a	8.36 ± 8.28 a
19 June	1.	3.22 ± 1.41 a	7.72 ± 2.41 a
	2.	2.63 ± 1.56 a	5.63 ± 3.59 b
	3.	3.13 ± 1.78 a	9.04 ± 8.34 ab
17-21 September	1.	4.9 ± 1.37 a	8.71 ± 2.83 a
	2.	4.54 ± 1.29 ab	8.04 ± 2.4 a
	3.	3.68 ± 1.46 b	13.91 ± 8.55 b

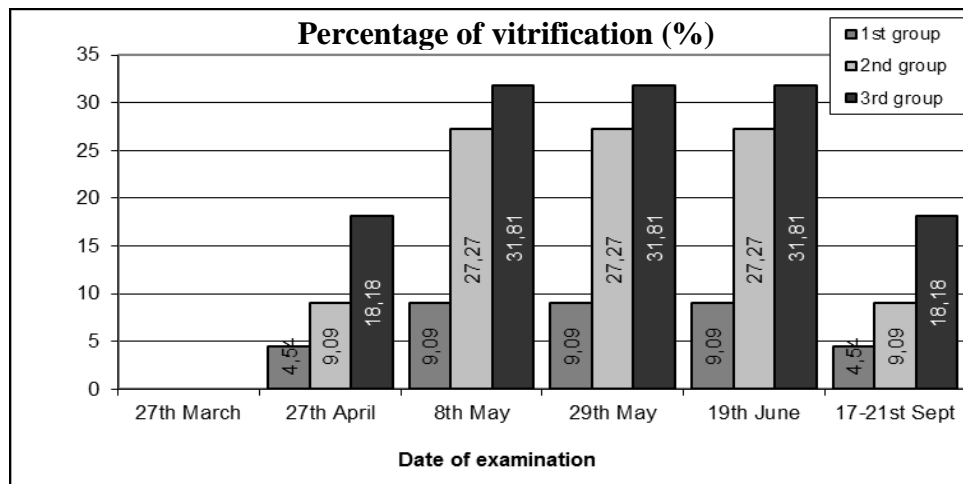
The number of roots was the highest in group 3 during almost every measuring times except March. Averagely and (as compared with the other 2 group) significantly the most (13.9) roots were developed in the group 3 (after the 34 day period of pre-treatment). Length of roots was measured only at the last evaluation. There were not positive correlation between the length of pre-treatment and root, the longest (41.19 mm) roots were found in group 1.

All bulbs (100%) differentiated roots in the first and second group. In the case of group 3, rooting rate was maximum 77.27% from May (during March and April, this stock produce roots the least of all). PB decreased rooting because of the over-long period of pre-treatment (figure 15).



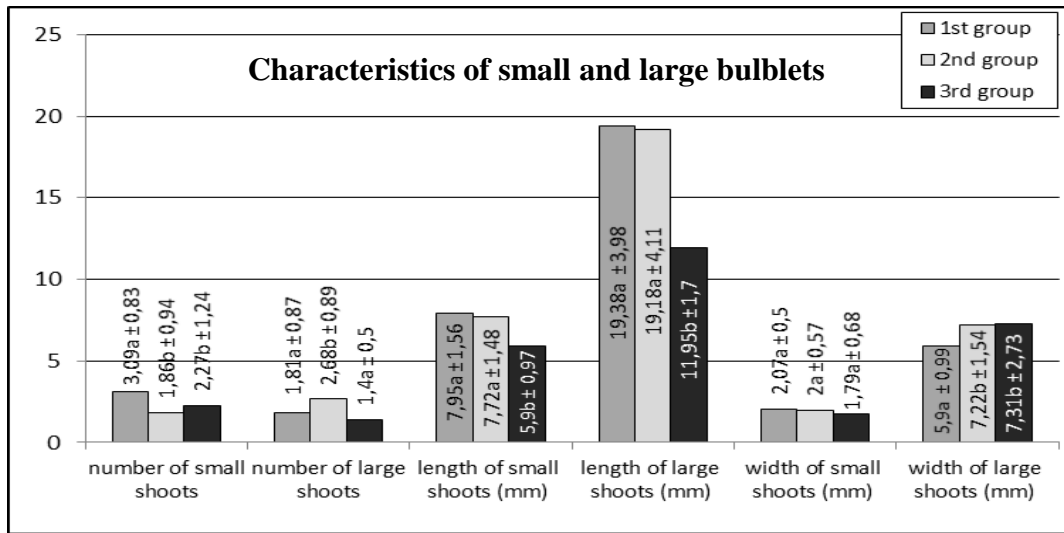
**Figure 15:** Rooting percentages of *in vitro* *Narcissus angustifolius* in different groups and examination times

First time, hyper hydrated (vitrificated) bulbs (with the largest ratio: 18.18%) were found on 27 April in the 3<sup>rd</sup> group. At the next evaluation (on 8 May) about every group, higher percentages of vitrification (maximum 31.81% in the group 3) were observed. By the last examination (in September), vitrification decreased unto 18.18% (3<sup>rd</sup>), 9.09% (2<sup>nd</sup>) and 4.54% (1<sup>st</sup> group) (figure 16).



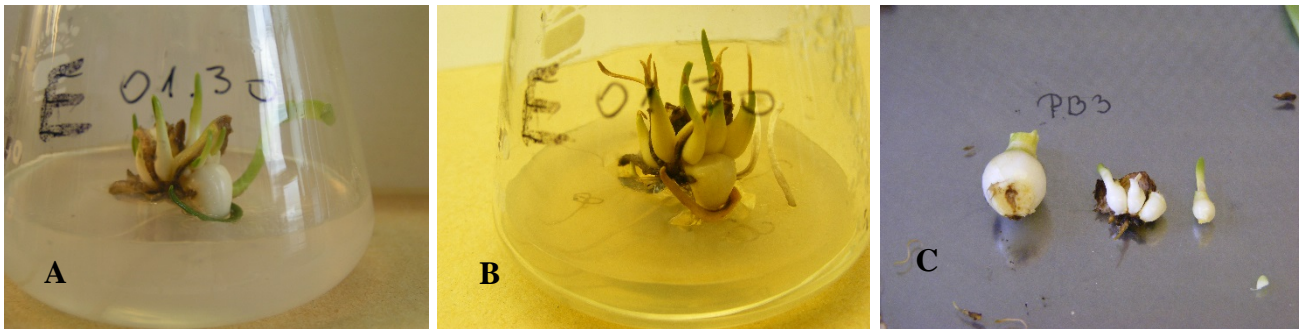
**Figure 16:** Percentage of hyper hydrated *in vitro* *Narcissus angustifolius* bulbs in different groups and examination times

At the last examining (on 21 September), bulblets were separated according to their sizes (small and large ones, figure 17). Averagely and (compared to the other 2 groups) significantly the most small (3.09) and large (2.68) bulblets were found in the 1<sup>st</sup> and 2<sup>nd</sup> group. Both of small (7.95 mm) and large (19.38 mm) bulblets elongated mostly in group 1, and significantly shorter and narrower bulblets were differentiated in the 3<sup>rd</sup> (in proportion to the other 2 group). Also, width of small bulblets was (not significantly) the largest (2.07 mm) in the 1<sup>st</sup> stock, and similar result (of large bulblets) was detected in the case of group 3 (7.31 mm).

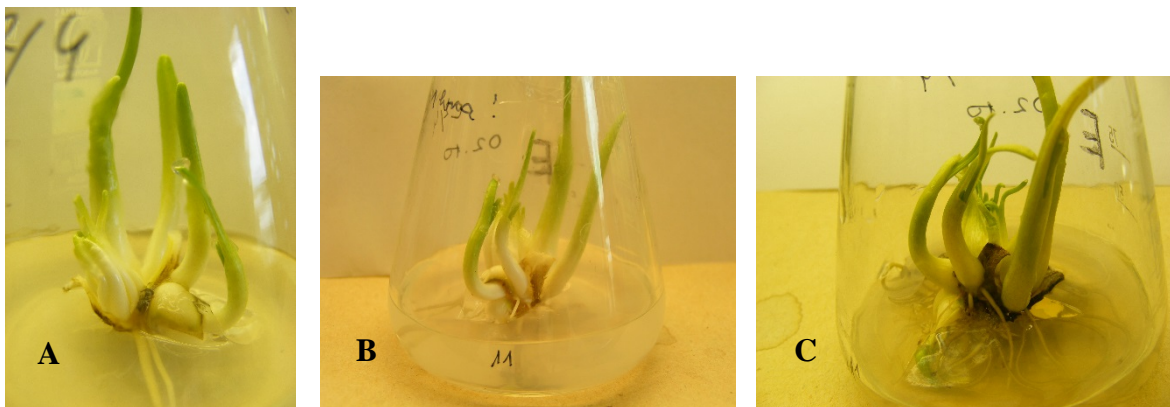


**Figure 17:** Characteristics (number, length and width) of small and large *in vitro* *Narcissus angustifolius* bulbets on 21 September  
 (\*: small: shorter than 9mm, large: longer than 10 mm)

The same individual was photographed different times (figure 18-20). On the latest one, vitrified, abnormal bulbets were shown.

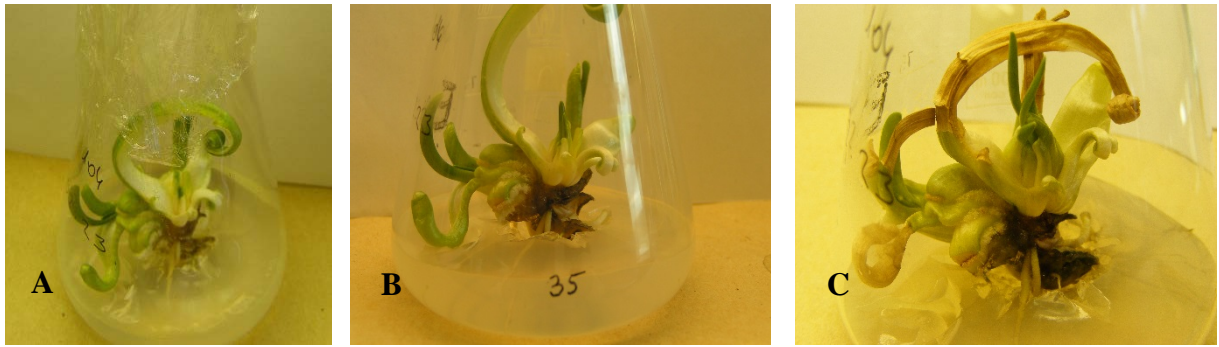


**Figure 18:** Specimen No. 1 (in the 1<sup>st</sup> group) on 27 March (A), 29 May (B) and 17 September (C)



**Figure 19:** Specimen No. 11 (in the 2<sup>nd</sup> group) on 8 (A) and 29 May (B) and 18 September (C)



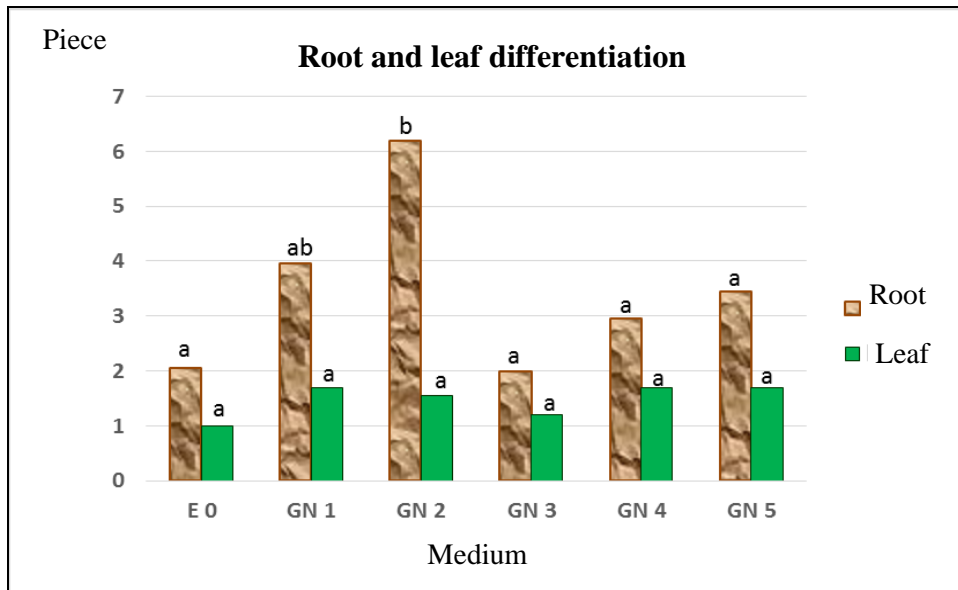


**Figure 20:** Specimen No. 35 (in the 3<sup>rd</sup> group) on 27 March (A), 29 May (B), and 19 September

### 3.2.3. The effect of media (during rooting)

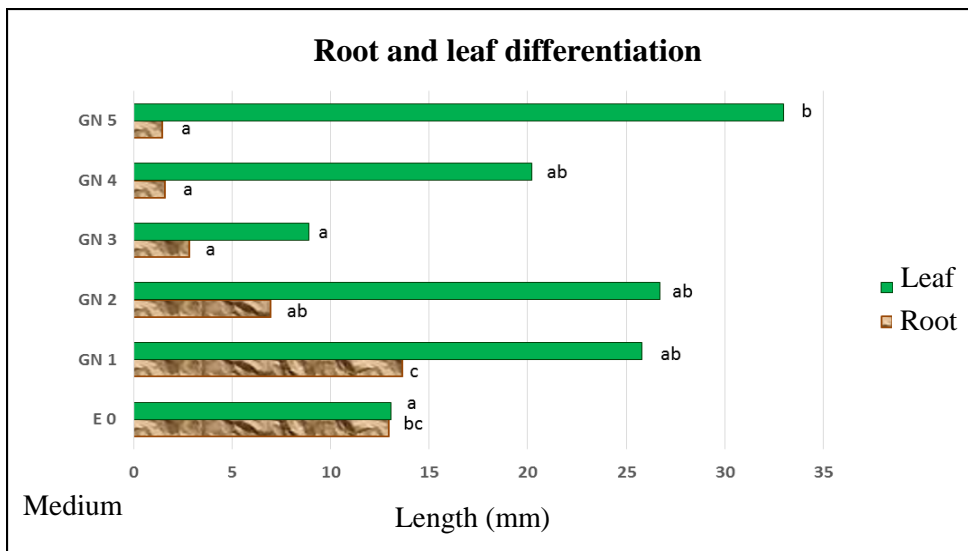
During rooting, 6 kind of medium was used (table 3). I supposed that PB stimulate rooting, as I experienced in the previous (multiplication) trials (when rooting was unwanted).

As shown on Figure 21, averagely and mostly significantly the highest number (6.2) of roots was achieved on medium GN2. If only NAA was added to the medium (GN1, GN2), more shoots were found than in the case of control and medium with PB (GN3, GN4) or PB + NAA combination. As compared with the other medium, almost in every cases (except E0 control: 12.95 mm) significantly the longest roots (13.56 mm) were observed on GN1.



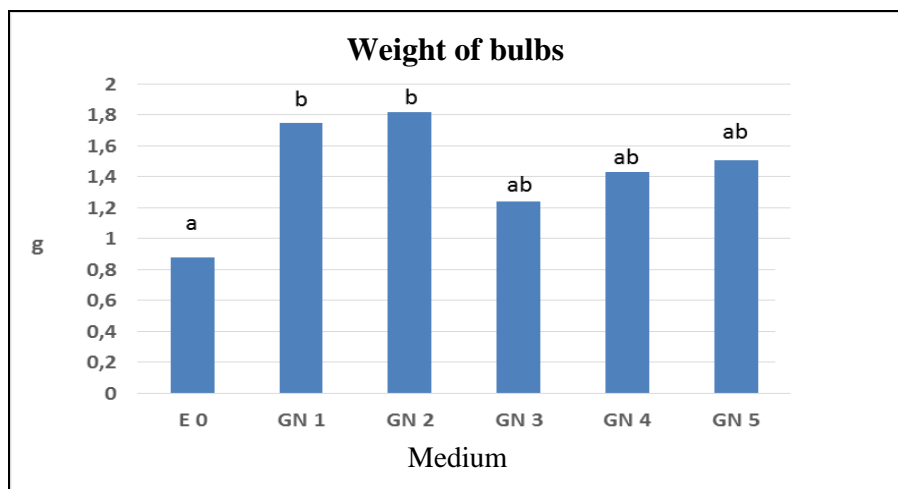
**Figure 21:** Average number of roots and leaves on rooting media

There were not significant differences between the numbers of leaves (figure 21), bulbs developed averagely 1.7 leaves on GN1, GN4 and GN5 medium. The longest (32.99 mm) leaves were found on GN5 and significantly shorter ones (8.88 mm) were obtained on GN3 (Figure 22).



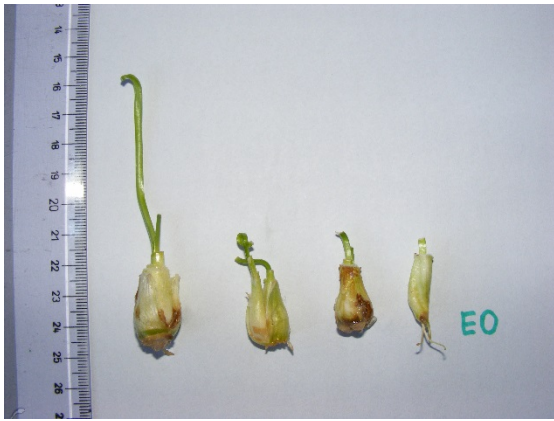
**Figure 22:** Average length of roots and leaves on rooting media

Average bulb weights on different media were presented on figure 23. The best results were also achieved on medium supplemented with NAA: 1.82 g (GN2), 1.75 g (GN1), 1.51 g (GN5, which contained NAA + PB). Significantly the easiest (0.88 g) bulbs (figure 24) were found in the case of control E0 medium (as compared with results of GN1 and GN2).



**Figure 23:** Average weight of bulbs on rooting media

Healthy, large, well-rooted bulbs developed on media with solely NAA (GN1, GN2, Figure 25-26), not so in the case of GN4 and GN5 (with PB). Probably abnormal, twisted leaves (figure 28) indicated the stress which was caused by higher concentration of PB. Combination of PB + NAA (GN5) was not efficient for bulb-growth as pale green, twisted, elongated leaves were shown (figure 29).



**Figure 24:** Bulblets from medium E0



**Figure 25:** Bulblets from medium GN1



**Figure 26:** Bulblets from medium GN2



**Figure 27:** Bulblets from medium GN3



**Figure 28:** Bulblets from medium GN4



**Figure 29:** Bulblets from medium GN5

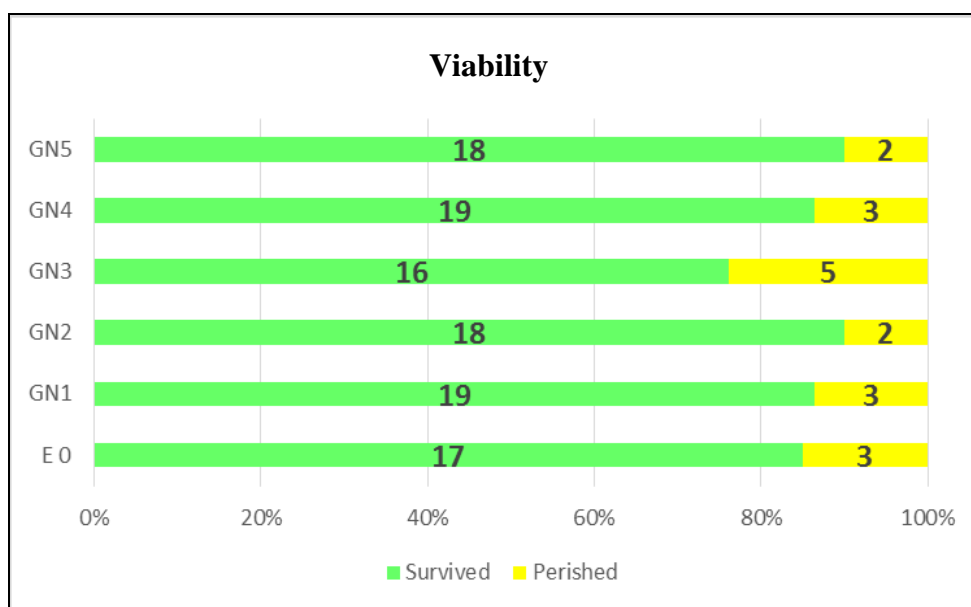
### 3.3. Results of acclimatization

*Narcissus angustifolius* has only 3 leaves (it is a typical characteristics of the species), so there were not significant differences between the leaf-numbers of acclimatized plants. Nevertheless, the most leaves (2.68) were achieved (on 14 May) as after-effect of medium GN1. At the same time, and compared to the (after-effect of) medium GN3, significantly longer leaves were found on plants from GN1 (table 10).

**Table 10:** Number and length of leaves of acclimatized plants

Medium	Examination time	Leaf number	Leaf length (mm)
E 0	<i>16 April</i>	1.18 ± 0.53 a	1.28 ± 1.13 a
	<i>14 May</i>	1.82 ± 1.81 a	1.46 ± 1.17 ab
	<i>25 June</i>	1.24 ± 0.83 a	1.54 ± 1.57 a
GN1	<i>16 April</i>	1.74 ± 0.87 a	1.87 ± 1.62 a
	<i>14 May</i>	2.68 ± 1.11 a	2.67 ± 2.14 a
	<i>25 June</i>	1.42 ± 0.61 a	1.76 ± 1.37 a
GN2	<i>16 April</i>	1.72 ± 0.67 a	1.18 ± 0.51 a
	<i>14 May</i>	1.67 ± 1.28 a	1.91 ± 2.27 ab
	<i>25 June</i>	1.00 ± 0.97 a	1.04 ± 1.20 a
GN3	<i>16 April</i>	1.44 ± 0.81 a	0.92 ± 0.82 a
	<i>14 May</i>	2.06 ± 1.98 a	0.86 ± 0.84 bc
	<i>25 June</i>	1.19 ± 0.75 a	1.47 ± 1.69 a
GN4	<i>16 April</i>	1.74 ± 0.99 a	1.25 ± 0.80 a
	<i>14 May</i>	2.58 ± 1.35 a	1.95 ± 1.25 ab
	<i>25 June</i>	1.21 ± 0.92 a	1.28 ± 1.42 a
GN5	<i>16 April</i>	1.39 ± 0.70 a	1.25 ± 0.75 a
	<i>14 May</i>	2.11 ± 1.49 a	1.38 ± 0.97 ab
	<i>25 June</i>	0.78 ± 0.81 a	1.07 ± 1.27 a

Examining viability, there were not considerable differences between the after-effects of rooting media (except GN3, where survival rate decreased below 80%). In the case of all medium, 86% of plants successfully survived acclimatization (figure 30).



**Figure 30:** Viability (percentage and number of survived plants) at the end of acclimatization

### 3.4. New scientific results

Complete micropropagation technology of *Narcissus angustifolius* was processed, including:

- Sterile cultures were produced and *in vitro* propagation was started with combination of PB and BA.
- BA + PB combination resulted in higher multiplication rate than BA (during *in vitro* propagation).
- The number of differentiated large bulblets was increased with BA + PB combination during *in vitro* propagation.
- Vitrification (with an optimal multiplication rate) was decreased during shoot-induction trial.
- Accessories of optimal rooting medium were ascertained.
- Paclobutrazol was firstly used during micropropagation of daffodil.

## 4. CONCLUSIONS, RECOMMENDATIONS

### Starting of culture

Bulbs (which were sterilized with the use of 70% ethanol and 1% HgCl<sub>2</sub> on 20 July 2009) seemed to be healthy after 1 week, but some of them were infected by Gram-negative bacterium, so the first 5 bulbs were perished. Survived bulbs were soaked in solution supplemented with 250 mg/l Cefotaxime and malachite. After then (under growth regulators: 1 mg/l BA + 0.1 mg/l NAA) 8 explants (bulb-scales) were proved to be sterile, and 6 of them developed shoot very slowly.

In conclusion after the first starting, conventional sterilization proved to be unsatisfying. Furthermore, bacterially infected bulbs were successfully decontaminated with the use of 250 (solidified) or 200 mg/l (liquid medium) Cefotaxime and the number of bulblets could be expanded 10 fold during 1.5 year. In spite of it, first starting produced few sterile bulbs so this procedure was repeated.

During second starting, sterilized (uncut) bulbs were placed on incubation media, and later 6 remained, healthy bulbs were cut and transferred to 6 kinds of starting media. Consequently, BA (which was added for incubation medium) has not affected the number of shoots during starting (even though PB was used in the next step). Then again if PB (in 2.5 mg/l concentration and with 1 mg/l BA) was used after hormone-free treatment, shoot number was significantly increased to 8.7. If only BA was used alone (both in media for incubation and starting), only 2.1 shoots were achieved. As unwanted side-effect, explants produced roots (I had to cut them during transferring). Vitrified cultures were also detected (in low rate), and these specimens were placed on hormone-free medium in order to dissolve this malformation.

On the whole, *in vitro* starting *Narcissus angustifolius* is difficult, but foremost, during this process (after hormone-free pre-treatment as incubation) PB + BA combination was successfully enhanced multiplication rate 4 fold than in the case of using only BA.

### **Multiplication of cultures**

#### **The effect of benziladenine and naphthalene acetic acid on the multiplication**

All told about this first multiplication trial, few shoots developed on ½ MS medium with 1 mg/l BA + 0.1 mg/l NES, although similar concentration of BA + 0.2 mg/l NAA proved to be better (averagely 3.5 small shoots differentiated in this case). 0.3 mg/l NAA (E30) was not optimal, shoot number decreased to 0.76 on this medium, and some cultures did not produce small shoots. In point of the number of large shoots, E20 medium (containing 0.2 mg/l NAA) was also the best (2.03 and 1.71), although there were not significant differences between values. Higher ratio (13.3 and 28.8%) of vitrification was observed on E30 medium, so not only too high BA level, but also similarly overdosed auxin could result vitrification. On this latter medium 6 cultures perished (probably due to the vitrification). Rooting was noticed (as an unwanted side-effect), it probably caused by the length of culture time (and not concentration of auxin). *Narcissus angustifolius* produced shoots slowly therefore long *in vitro* culture period was necessary.

As this species has not micropropagated yet, so own results could be compared with literary information of other *Narcissus* taxa.

In GEORGE's (1996) opinion, 2 mg/l BA + 1 mg/l NAA was optimal for daffodils' *in vitro* propagation, although cultivars multiplication was carried out easier than in the case of wild species, because higher concentrations of BA caused vitrification in the latter cultures. During the trial of *Narcissus bulbocodium* species (SANTOS et al., 1998), 2-4 mg/l BA + 1 mg/l IAA combination was optimal. More daffodil cultivars were *in vitro* propagated (on MS basic medium supplemented with 0.5-1 mg/l NAA and 2 mg/l BA) by SOCHACKI and ORLIKOWSKA (2005). A 24-week-period was necessary for differentiation of new bulblets. During this long time, cultivar 'Carlton' produced only 8.4, and the other one ('Hewelius') developed 15.2 bulblets. As they experienced, rate of multiplication (during equal conditions) was strongly depended on the cultivar (genotype). During the study of HE at al. (2005), development of the technology of *in vitro* propagation of *Narcissus tazetta* var. *chinensis* 'Huanghua' and 'Nanridao' was aimed. 1 month before this trial, explant was prepared and refrigerated (4 °C). For bulb induction, MS medium contained 0.5 mg/l BA and 2 mg/l NAA. ZAHRA and ORAN (2009) micropropagated a species named *Narcissus tazetta* on MS basal medium supplemented with different concentrations of BA, NAA. During multiplication, the best result (rate: 3.4, without vitrification) was achieved in the case of 10 mg/l BA + 0.5 mg/l NAA. SUN et al. (2010) worked out the micropropagation of *Narcissus* 'Arkle' with the use of

bulbs scales explants. For starting 3 mg/l BA + 0.5 mg/l NAA + 0.2 mg/l IAA, for multiplication 1.5 mg/l BA + 0.3 mg/l NAA proved to be the best. All in all, optimal type and concentration (and cytokinin-auxin rate) was depended on the actual daffodil species or cultivars.

### **The effect of paclobutrazol on multiplication**

On the whole (bring in vitrification), PB4 medium supplemented with 0.25 mg/l PB, 1 mg/l BA and 0.1 mg/l NAA was the best for multiplication. This PB dose is lower than the other optimal levels which mentioned in the case of bulb plant relatives.

JEVCSÁK et al. (2012) combined BA and PB during micropropagation of *Leucojum aestivum*. The best result (7.8 large shoots) was obtained on medium with 0.5 mg/l BA + 0.5 mg/l PB. Enhanced PB concentration (2.5 mg/l) decreased shoot number (to 6.4). No vitrification but 100% rooting was observed. BA + PB combination was applied by MOSONYI et al. (2013) in the case of *in vitro* propagation of snowdrop (*Galanthus nivalis*). The optimal was 0.5 mg/l BA + 2.5 mg/l PB (15.08 bulblets), but only 10.27 bulblets were found when 0.25 mg/l PB was used with 0.5 mg/l BA. In the case of the best shoot reproduction, 20% vitrification and 8% rooting was achieved.

During multiplication of *Narcissus angustifolius*, the second best medium contained similar concentration of PB and half level of BA. The top 2 medium (PB4 and PB3) resulted 7.8 and 6.7 small, and 2.5 and 1.2 large bulblets. Vitrification was only 10% in the case of PB4.

As compared with the previous trial, PB significantly enhanced shoot differentiation (4 small bulblets were found, that was double more than reached on optimal medium of earlier trial). However, vitrification was occurred especially in the case of higher PB concentrations. Rates of this malformation were similar on optimal media of this (0.25 mg/l PB + 1 mg/l BA + 0.1 mg/l NAA) and previous. PB stimulated rooting (as undesired side-effect during multiplication), and 70% of individuals developed roots on the optimal PB 4 medium. Furthermore, this ratio was about 50% in the previous trial. PB is recommending as an *in vivo* root stimulator, probably this agent also result higher rooting percentages during *in vitro* propagation. Although in this trial rooting is not desire (before the next multiplication I had to prune all roots from the bulbs), PB found ideal by reason of the enhanced shoot differentiation.

### **The effect of 2-izopentenyl-adenin on multiplication**

During this trial, the effect of 2 kind of cytokinins (BA and 2iP) and PB was examined in different combinations. The aim of this study was to notice the effect of 2iP on shoot differentiation and vitrification. The numbers of small and large shoots were separately evaluated, but summarized results were presented in this chapter. In the case of 2iP + PB combination, the multiplication rate was only 1.9, while the same level (1 mg/l) of 2iP + BA result higher rate (2.25). 2iP + (0,5 or 1 mg/l) BA + 0,25 mg/l PB enhanced this value to 5.25

and 5.9, but in these cases (on both medium) 100% vitrification was occurred. In spite of it, the combination of 2 cytokinin and PB proved to be the best during this trial. Bulblets with worse conditions (which caused by vitrification) were also observed on medium N5 and N6. To decrease this malformation, transferring and keeping on hormone-free medium is suggested.

In conclusion, 2iP was not efficient for shoot differentiation (few shoots were found, though ratio of vitrification was also low). Combination of 2iP + BA + PB enhanced the number of shoots, but not as much as I achieved in the previous trial on medium with BA + PB. *Narcissus angustifolius* seemed to be very sensible for different growth regulators, and only one type of cytokinin is recommended to combine with PB.

On the other hand, snowdrop (*Galanthus nivalis*, belonged to a close relative genus) developed more shoots (with a multiplication ratio 3.7) on medium containing 1 mg/l 2iP + 0,2 mg/l IAA (TILLY-MÁNDY et al., 2006), while 2 mg/l BA effected only 2.6. Consequently, *Narcissus angustifolius* and *Galanthus nivalis* had got different reaction to this cytokinin.

#### **The effect of 6-benzil-amino-purin-ribozid on multiplication**

The aim of this trial was to put BA-ribozid (BAR) to the proof. BAR was described as an efficient cytokinin which cause less vitrification.

DOBRÁNSZKI et al. (2000a) used 1 mg/l BAR instead of BA in the case of apple rootstocks MM 106 and JTE-H. During adventitious shoot formation of another apple rootstock (M 26) 5 mg/l BAR proved to be optimal (DOBRÁNSZKI et al., 2004). Similar result (27.4 shoots of *Rubus fruticosus* L. 'Loch Ness') was achieved with the use of BAR in the highest (1,59 mg/l) concentration (MAGYAR-TÁBORI et al., 2014). In the case of micropropagation of hortensia, 2 mg/l IAA + 0.2 mg/l BAR combination was recommended by TÓTH and KISS (2005). I have not found information about BAR application in line with daffodils and other bulb plants.

In this study, bulblets were separated according to their sizes. In the case of large bulblets, BR1 medium proved to be the best (4.2), and the second was BR2 (3.5). On the other medium, more BAR concentration increased the number of large bulblets. Examining the small bulblets, the healthiest and highest number (3) of bulblets were also observed on medium BR1 (and control). Higher dose of hormone enhanced vitrification especially in the case of BR5 and BR6 medium (some cultures perished on these media).

Unfortunately, rooting was also occurred (as an unwanted side-effect). The most (but short) root developed on medium BR3 and BR4 (6.6 and 6.4 piece). On the other hand, BR1 (which was the best shoot-stimulator) medium resulted only 3.2 (highly elongated) roots. Rooting is attributable to the use of PB (not more than 50% of cultures differentiated roots on hormone-free medium). Nevertheless, few root is optimal during multiplication.



As compared with my previous and actual results of BAR, induction medium with this cytokinin (which was firstly used during *in vitro* propagation of *Narcissus*) and after-culturing on hormone-free medium were ideal: averagely little more than 7 shoots developed. (More than I reached as the highest shoot-number in the previous trial.) This positive effect was shown on enhanced number of large shoots.

### **The after-effect of paclobutrazol**

Concrete data are not available in the literature concerning PB application and its effects in the micropropagation of *Narcissus angustifolius*, so inferences were drawn from my experiences.

This half-year-long trial was based on the length of pre-culturing on medium with PB + BA + NAA. Furthermore, ratio of vitrification was also investigated in according to the different periods of pre-treatments (11, 23 and 35 days – namely group 1, 2 and 3). The highest number of shoots was found in group 2, additionally the length and number of roots were increased in every stocks. In the case of 1<sup>st</sup> and 2<sup>nd</sup> culture period, similar results were observed (at the last examination time), but there were differences between the numbers of small and large shoots. In group 1, higher number of small (3.1) and less large (1.82) shoots developed, while in the case of 2<sup>nd</sup> stock, the number of small shoots decreased (to 1.86) and large ones increased (to 2.7). All in one, significant differences were not detected between shoot numbers of group 1 and 2, although the best results was observed in the latter stock accordingly the size of shoots. Significantly longer shoots were found in the first 2 group than the 3<sup>rd</sup>, in point of both small (8.05 and 5.91 mm) and large shoots (19.41 and 11.95 mm). Similar tendency was noticed during evaluation of shoot width, but not in the case of (low) values of rooting. Optimally few and short roots were found in this trial (and specimens with this kind of roots can handle easier and quicker, so less workforce is enough). In conclusion, cultures had a good reaction to PB, and root differentiation was decreased in group 2.

Vitrification was successfully reduced. At the last examination vitrification ratio was only 4.54% (group 1) and 9.09 % (group 2). The 3<sup>rd</sup> period proved to be too long, 18.18% of individuals' vitrified. Nevertheless, these values were significantly smaller than which were experienced during previous trials. Summarizing, the second period of incubation (23 days) was the best, because of the low vitrification and (chiefly) the higher number of large bulblets.

### **Rooting**

In the case of *in vitro* rooting of daffodils, only few and disparate data (from 2 article) were available about *N. tazetta* (a related species). As HE at al. (2005) experienced, optimal was ½ MS medium with 0.05 mg/l NAA for *in vitro* rooting of *N. tazetta* var. *chinensis* 'Huanghua' and 'Nanridao' cultivars. In another study (ZAHRA and ORAN, 2009) with the same species, the

best result (only 1 root) was achieved on medium containing 0.6 mg/l NAA, but higher doses of this auxin were ineffective.

In my trial, PB was also added to the medium (because this agent effectively stimulated rooting during multiplication), but the best result (averagely 6.2 root) were obtained with the use of 0.2 mg/l NAA. Only 3 roots developed on medium with the same concentration of PB, and combination of this 2 growth regulators decreased rooting. As compared with the other treatments, significantly heavier bulblets (1.7-1.8 g) were found on media supplemented with NAA.

PB positively stimulated *in vitro* rooting during some study. For example PB resulted significantly more and shorter roots in the case of *Tibouchina urvilleana* (KOZAK (2006), stronger root system of *Chrysanthemum* cultivar 'Ludo' (KUCHARSKA és ORLIKOWSKA (2008) and enhanced rooting of *Syringa x hyacinthiflora* 'Luo Lan Zi' (HONGXIA et al. (2009) and *Galanthus elwesii* 'Hook' (MOSONYI et al. (2013). But in my trial PB was not efficient as root stimulator (if the main aim was specifically *in vitro* rooting of *Narcissus angustifolius*), the best was 0.2 mg/l NAA.

### **Acclimatization**

Importantly of acclimatization, roots of all plants (originated from every rooting media except GN5 which contained PB + NAA) grown out of the bottom of containers. If only one growth regulator was used for rooting medium, significantly better results were achieved, although the survival rate (averagely 86%) was not affected by compounds of rooting media.

## **Recommendation (complete micropropagation technology of *Narcissus angustifolius*)**

### **1. Sterilization**

Surface-sterilization: first, wash the bulbs under running tap water (and add some drops of Tween-80). After then, soak them in 70% ethanol (10 minutes) and 1 ‰ HgCl<sub>2</sub> (10 minutes), and rinse bulbs in distilled water. Afterward, rotate cultures in liquid solution with 250 mg/l Cefotaxim + 3 mg/l malachite (bactericides) until 1 week. Solidified medium with 200 mg/l Cefotaxime is also suitable.

### **2. Incubation**

Put the uncut bulbs on hormone-free S medium containing BM (JÁMBORNÉ BENCZÚR and MÁRTA, 1990) macro- and HELLER (1953) microelements and store them in refrigerator (4°C as vernalization) 6 weeks.

### **3. Starting**

Place the sterile, sliced bulb on ½ MS basal medium with 2.5 mg/l PB + 1mg/l BA. This procedure need 4-5 month because of the slow regeneration. In the case of occurring

vitrication, put vitrificated specimens on medium without hormones and keep them until further 4-5 months.

#### **4. Multiplication**

Place the (sliced) bulbs on ½ MS (MURASHIGE and SKOOG, 1962) basal medium in 2 compounds according as our aim to generate small or large bulblets. For developing more small bulblets 0.25 mg/l PB + 1 mg/l BA + 0.1 mg/l NAA (PB4) medium is recommend. BR1 (0.1 mg/l PB + 0.5 mg/l BAR + 0.1 mg/l NAA) is better for differentiation of large bulblets. Unwanted vitrication and rooting can appear during multiplication, so after this period (duration: some months) put in a hormone-free phase (with similar length of time) in order to decrease and eliminate vitrication.

To short the whole procedure (just half year in total) keep the *in vitro* stocks previously on medium with growth regulators (BA or BAR + PB + NAA) until 23 days (or so 3 weeks) and then on hormone-free medium till some months. Choose this method if higher number of large bulblets (with decreased rooting and vitrication) is desirable.

#### **5. Rooting**

For rooting (unto 3 month), keep the uncut bulbs on ½ MS basal medium containing 0.1 mg/l NAA (3 month).

#### **6. Acclimatization**

Plant the rooted bulbs in a 1:1 mixture of peat-perlite and acclimatize them progressively unto 1.5-2 (3) month. Optimal timing is important: after the end of acclimatization, bulbs must plant out (to open ground).

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## 5. PUBLICATIONS IN RELATION TO THE PhD THESIS

### 5.1. Publications

#### *Publications without IF*

**Jevcsák M., Ördögh M., Kohut I., Jámborné Benczúr E.** (2012): A különböző auxinmennyiségek hatása a *Narcissus poeticus* ssp. *radiiflorus* mikroszaporítása során. Budapest. Kertgazdaság 44. (1) 59-63.

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**Jevcsák M., Ördögh M., Kohut I., Jámborné Benczúr E.** (2013): A paclobutrazol hatása a *Narcissus poeticus* ssp. *radiiflorus* in vitro tenyészetekre. Budapest. Kertgazdaság 45. (1) 29-34.

**Jevcsák M., Ördögh M., Jámborné Benczúr E.** (2014): Különböző növekedésszabályozók hatása a *Narcissus poeticus* ssp. *radiiflorus* in vitro gyökeresedésére. Budapest. Kertgazdaság 46. (3) 36-43.

**Jevcsák M., Ördögh M., Jámborné Benczúr E.** (2015): The after-effect of paclobutrazol on morphological characteristics of *in vitro* *Narcissus poeticus* ssp. *radiiflorus* plants. Budapest, International Journal of Horticultural Science, 21. (1–2): 43-46.

## 5.2. Proceedings

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**Jevcsák M., Jámborné Benczúr E., Kohut I., Komendár V.** (2011.): Különféle kezelések hatása a *Narcissus poeticus* ssp. *radiiflorus* steril tenyészetének indítása során. Erdei Ferenc VI. Tudományos Konferencia. III. kötet. Augusztus 25-26., Kecskemét. 339-343.

**Jevcsák M.** (2013): Különböző táptalajok hatása a *Narcissus poeticus* ssp. *radiiflorus* *in vitro* szaporítása során. PhD-konferencia 2013, Balassi Intézet (és Márton Áron Szakkollégium), február 8., Debrecen. 85-94.

### *Conference papers (Hungarian, abstracts)*

**Jevcsák M., Jámborné Benczúr E., Kohut I., Komendár V.** 2011: Előzetes eredmények a *Narcissus poeticus* ssp. *radiiflorus* termesztésbe vonásához. XVII. Növénynevelési Tudományos Napok, 2011. április 27. Óvári Judit (szerk.): Összefoglalók. MTA Agrártudományok Osztályának Növénynevelési Bizottsága, Magyar Növénynevelők Egyesülete, MAE Genetikai Szakosztálya. 113.

**Jevcsák M., Kohut I., Ördögh M., Jámborné Benczúr E.** 2012: A *Narcissus poeticus* ssp. *radiiflorus* termesztésbe vonásának elindítása mikroszaporítás segítségével. XVIII. Növénynevelési Tudományos Napok, 2012. március 6. Veisz Ottó (szerk.): Összefoglalók. MTA Agrártudományok Osztályának Növénynevelési Tudományos Bizottsága, Magyar Növénynevelők Egyesülete, MAE Genetikai Szakosztálya. 91.

### *Conference papers (international, full paper)*

**Jevcsák M., Höhn, M., Benczúr, E., Jevcsák Sz., Komendar, V.I.** 2010. *Narcissus poeticus* ssp. *radiiflorus* area scientifically based protection and restitution in the Ukrainian Carpathians. Bicentenary of vegetation cover studies of the Carpathians: Proceedings of International Scientific Conference devoted to the 130<sup>th</sup> anniversary since Antal Margiittai's birthday (2010 September, 16-18, Munkachevo-Beregovo-Ukraine). 72-75.

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*Conference papers (international, abstracts)*

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