Improved regeneration of eggplant doubled haploids from microspore-derived calli through organogenesis

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Key message:

We report on an improved process of doubled haploid plant regeneration starting from eggplant androgenic calli obtained through isolated microspore culture.

Abstract

Doubled haploid (DH) technology allows for the production of pure lines, useful for plant breeding, through a one-generation procedure that reduces considerably the time and resources needed to produce them. Despite the advantages of microspore culture to obtain DHs, this technique is still insufficiently developed in eggplant, where DHs are produced from microspore-derived calli through organogenesis. At present, very little is known on the best *in vitro* conditions to promote this process. This is why in this work

we addressed the optimization of the process of regeneration of eggplant DH plants from microspore-derived calli. We evaluated the effect of different media compositions in the induction of organogenesis, in the promotion of shoot growth and elongation, and in root growth. According to our results, we propose the repeated subculture of the calli in MS medium with 0.2 mg/l IAA and 4 mg/l zeatin to produce shoots, and then the repeated subculture of the excised shoots in basal MS medium to promote their conversion into entire plantlets. This procedure yielded 7.6 plants per 100 cultured calli, which represents a ~4x increase with respect to previous reports. We also evaluated by flow cytometry and SSR molecular markers the effect of these *in vitro* culture conditions in the rate of DH plant production, finding that ~70% of the regenerated plants were true DHs. These results substantially improve the efficiencies of DH recovery published to date in eggplant, and may be useful to those working in the field of eggplant doubled haploidy and breeding.

Abbreviations:

BA: 6-Benzyladenine

DH: doubled haploid

GA_3: Giberellic acid

IAA: indole acetic acid

MDE: microspore-derived embryo

Production of DHs reduces the time and costs required to produce homozygous, pure lines, essential for hybrid seed production. This is why there is an increasing effort to extend this technology to new species and to optimize the protocols already existing for agronomically interesting crops (Asif et al. 2014; Castillo et al. 2014; Eshaghi et al.

2015; Kim et al. 2013; Parra-Vega et al. 2013). In eggplant, DHs are typically produced through anther culture. This approach, however, has several limitations, including the possible occurrence of somatic regenerants derived from anther walls, the uncontrolled contribution of tapetal cells to culture conditions, and the general low efficiency of the technique (Seguí-Simarro et al. 2011; Seguí-Simarro 2015). To avoid these problems, it is possible to perform isolated microspore culture, which is much more efficient (Corral-Martínez and Seguí-Simarro 2012, 2014). Unfortunately, microspore culture in eggplant has a bottleneck still to be solved, which is the arrest of the globular-to-heart-shaped embryo transition, producing undifferentiated calli (Corral-Martínez and Seguí-Simarro 2012). This implies that DHs must be obtained through organogenesis from the microspore-derived calli. Although the first part of the process (induction of microspore embryogenesis) is highly efficient, little progress has been made in the second part (organogenesis from calli). In literature, different authors working with different eggplant explants proposed different protocols to regenerate plantlets (Franklin et al. 2004; Gisbert et al. 2006; Borgato et al. 2007; Xing et al. 2010; Kaur et al. 2011). Only two studies addressed the particular case of regeneration from microspore-derived callus (Miyoshi 1996; Corral-Martínez and Seguí-Simarro 2012), and their results were not conclusive. Therefore, the aim of this work is to optimize the process of regeneration of DH plants from androgenic calli obtained through isolated microspore culture.

We isolated and cultured microspores from ‘Bandera’ (a F1 hybrid from Seminis Vegetable Seeds Ibérica, S.A., Spain) according to the protocol described in Corral-Martínez and Seguí-Simarro (2012, 2014). ‘Bandera’ was selected because in previous studies, it showed the highest response among different eggplant genotypes (Salas et al. 2011; Corral-Martínez and Seguí-Simarro 2012). To promote organogenesis, calli

78 exceeding 1 mm after 30 days of culture were isolated and transferred to two modified
79 MS media (pH 5.8) previously described as useful to induce organogenesis in eggplant
80 calli, explants or protoplasts. One of them (M1) was supplemented with 2% sucrose,
81 0.8% plant-agar, 0.2 mg/l IAA and 4 mg/l zeatin (Miyoshi 1996), and the other (M4)
82 was supplemented with 3% sucrose, 0.4% Phytagel, 0.1 mg/l IAA and 2 mg/l zeatin
83 (Borgato et al. 2007; Xing et al. 2010). In our previous studies using 8 different media,
84 M1 and M4 produced the best results in terms of organogenesis induction (Corral-
85 Martínez and Seguí-Simarro 2012). Cultures were kept at 25ºC under a 12/12
86 photoperiod and subcultured every 5 weeks. Callus growth could be observed 4-5 days
87 after callus isolation. After 2 weeks of callus culture, the first organogenic buds were
88 formed on the callus surface (Figure 1A). After 5 weeks, we were able to discriminate
89 between two different types of calli. The first type appeared as compact and hard cell
90 masses, non friable, and sometimes greenish (Figure 1B). This kind of callus is, in
91 general, non organogenic (Seguí-Simarro and Nuez 2006; Corral-Martínez and Seguí-
92 Simarro 2012). We could confirm this since no further development was observed in
93 their surface. They eventually necrosed and died. The second type consisted of white,
94 spongy and friable masses. We frequently observed the formation of shoot primordia in
95 these organogenic calli, normally surrounded by two or more leaf primordia (Figure
96 1C). After 4 months of culture, calli in M1 produced 441 shoot primordia whereas calli
97 in M4 produced 26 shoot primordia. 157 out of the 1,392 calli cultured in M1 (11.3%)
98 produced at least one shoot primordium, while this rate was reduced to 16 out of 1,311
99 calli (1.2%) in M4. Most of the primordia produced were hyperhydrated and did not
100 develop further (data not shown). Differentiated shoots with an elongated stem, a
101 normal appearance, fully formed leaves, and no hyperhydration (i.e. ready for rooting;
102 Figure 1D) could only be observed in 51 out of the 2,703 calli (M1+M4). M1 was also

103 better than M4 in producing this type of shoot (37 vs 14 shoots, respectively). All these
104 results considered, M1 clearly appeared as better than M4 for organogenesis induction,
105 but none of the media tested was appropriate to promote shoot growth and elongation
106 efficiently. It is interesting to note that, as opposed to our results, Corral-Martínez and
107 Seguí-Simarro (2012) chose M4 instead of M1. This apparent discrepancy can be
108 explained by the time point used for the analysis. In the previous work, shoots were
109 studied after 5 weeks of culture (not after 4 months as hereby). Although M1 and M4
110 produced callus growth and shoot induction at a similar rate, M4 promoted rooting at a
111 higher rate. Nevertheless, the final percentage of entire DH plants was very low,
112 justifying the need for the improved regeneration protocol we hereby present.
113
114 To increase the rate of normal-appearing, elongated shoots, we tested the effect of
115 different media on shoot growth and elongation (Table 1) as a previous step before
116 rooting. We produced a new batch of 1,855 calli cultured in M1 medium. From them,
117 we excised 555 shoots with leaf primordia surrounding a visible meristem, and
118 transferred the shoots to four different media: M11, M12, M13 and M14. All of them
119 included MS medium (pH 5.8), 0.8% plant-agar and different growth regulators, as
120 follows. M11 included no regulators, M12 was supplemented with 0.3 g/l BA according
121 to Kaur et al. (2011), M13 was supplemented with 1.5 mg/l GA_3 (Shivaraj and Rao
122 2011) and M14 was supplemented with 1.5 mg/l GA_3 and 8 mg/l $AgNO_3$ (Xing et al.
123 2010). A minimum of 124 shoots were cultured in each of the four media. First, we
124 evaluated shoot elongation after 30 days of culture, classifying them in five discrete
125 types: (0) dead shoots; (1) no growth, or formation of swollen, hyperhydrated organs
126 with no stem growth (Figure 2A); (2) formation of new, no hyperhydrated organs, but
127 no stem elongation (Figure 2B); (3) formation of new, no hyperhydrated organs and

stem elongation (Figure 2C); (4) root formation in addition to all the features of type 3 (Figure 2D). Shoot development was found to be dependent on the elongation medium used according to a χ^2 test ($p \leq 0.05$). As seen in Figure 3, 61% of the shoots cultured in M11 formed new, normal-appearing organs (type 2, 3 and 4). From them, elongation was limited to 11% of the shoots, and formation of roots was observed in some cases (3%). In contrast, in GA_3-containing media (M13 and M14) the production of new organs was lower than in M11 (55 and 53%, respectively), although elongation was induced in a percentage of shoots similar to M11 (11%). In the particular case of M14, shoots elongated quickly, but many of the newly formed organs presented necrotic spots (Figure 2E). Overall, M12 was the worst medium in terms of promotion of shoot growth and elongation.

Non-rooting shoots were kept in their elongation media beyond the 30-day checkpoint, and individually transferred to rooting medium once they reached a minimal length of 2 cm. During the 6-month period considered, M13 was the medium that induced elongation in more shoots (Figure 4A; Table 1). Most of the non-rooted shoots developed within the first 3 months (Figure 4B). In particular, shoots in M14 reached the stage suitable for rooting faster than shoots from other media, including M13. Both media have 1.5 mg/l GA_3, known to be involved in many developmental responses, including the promotion of stem elongation (Moshkov et al. 2008). This would explain the higher shoot elongation in these media. M14 included also $AgNO_3$, an ethylene inhibitor known to promote shoot growth and elongation in several species (Mohinuddin et al. 1997; Sgamma et al. 2015; Zhang et al. 2001) including eggplant (Xing et al. 2010). However, the results of M14 after 6 months were clearly below those of M13. It is likely that the Ag^+ cations present in M14 had a toxic effect (Veen and van

153 de Geijn 1978) that accounted for the necrosed areas observed in these explants (Figure
154 2E), and prevented further development.
155
156 On the other hand, some shoots were able to form roots while they were cultured in
157 elongation medium, before the stem was 2 cm long, and therefore, before being
158 transferred to rooting medium. M11 induced rooting in 12.7% of the shoots, while in the
159 other media the frequency was much lower (Figure 4A; Table 1). These results pointed
160 to M11 (basal MS medium) as the best medium to induce the development of new
161 normal-appearing organs, including roots, and therefore the formation of entire
162 plantlets. Previous studies also pointed to this medium as the best to induce rooting in
163 different explants and calli of eggplant and wild relatives (Miyoshi 1996; Franklin et al.
164 2004; Gisbert et al. 2006; Xing et al. 2010). However, the frequency of rooting in M11
165 during the elongation period was relatively low (12.7%), possibly due to the fact that
166 most these shoots were not differentiated enough to be able to root spontaneously. This
167 is why we considered the addition of a rooting step (with M11) to the protocol. Around
168 71% of the elongated shoots produced in M11, M12, M13 and M14 developed roots
169 when transferred to M11, forming entire *in vitro* plantlets. As seen in Table 1, the
170 rooting percentages were remarkably similar, ruling out a different effect of the
171 elongation medium used in the rooting capacity of the shoots after transference to
172 rooting (basal) medium. Taken together, our short-term results (1 month) and mid-term
173 results (6 months) showed that M11 and M13 were the best performing media, and
174 while M13 produced more elongated shoots after 6 months, M11 produced more entire
175 plantlets. Besides, it appeared that the addition of an elongation step after shoot
176 initiation was beneficial not only to produce normal-appearing, elongated shoots, but
177 also to increase their rooting ability.

178

179 Rooted plants were transferred to pots with substrate and kept in a growth chamber at
180 25ºC and a 16/8 photoperiod (Figure 2F). For acclimatization and hardening, a
181 transparent plastic cup was placed covering the whole plant, and humidity was
182 progressively reduced by drilling the cup. Once plants were acclimated, their ploidy was
183 checked by flow cytometry. Small pieces of young leaves were chopped, processed
184 basically as described in Corral-Martínez and Seguí-Simarro (2012), and analyzed using
185 a Partec PA-I Ploidy Analyzer. Ploidy analysis of 88 microspore-derived plants
186 revealed that 5 (5.7%) were haploid, while 61 (69.3%) were diploid and 18 (20.5%)
187 showed higher ploidies. The histograms of 4 plants presented multiple peaks or
188 excessive background noise that precluded an unambiguous determination of their
189 ploidy. Diploid plants were analyzed with microsatellite (SSR) molecular markers using
190 the procedure and markers described in Corral-Martínez and Seguí-Simarro (2012).
191 These markers were proven heterozygous for the 'Bandera' donor plants. The 61 diploid
192 plants analyzed were consistently found homozygous for all the SSR makers used, as
193 expected since they came from isolated microspore cultures where anther wall tissue is
194 filtered out. These results are in agreement with our previous studies with these SSR
195 markers in 'Bandera', where we repeatedly proved a gametophytic (initially haploid)
196 origin for all the regenerants obtained (Corral-Martínez and Seguí-Simarro 2012).
197 Genetic variation, from gross ploidy differences to subtle genetic changes, is a well-
198 known phenomenon inherent to all *in vitro* culture processes (reviewed in Dunwell
199 2010). However, the consistency of the SSR analyses reported hereby and previously
200 published make us think that in this particular case, genetic variation should not have a
201 great impact. In addition, ~70% of DHs is a percentage better than previous results from
202 microspore cultures in this and other eggplant cultivars, which revolved around 60%

(Miyoshi 1996; Corral-Martínez and Seguí-Simarro 2012). This percentage is much better than those obtained through anther culture (46.4% of DHs in Salas et al. 2011, 25.6% in Rotino 1996, 23.5% in Tuberosa et al. 1987 and 15.4% in Dumas de Vaulx and Chambonnet 1982). Thus, it appeared that the prolonged *in vitro* culture time did not affect to the rate of occurrence of higher ploidies, while it seemed beneficial in terms of reducing the rate of haploids.

In this work we addressed the main limiting step of DH production in eggplant through microspore culture: the regeneration of entire DH plants from microspore-derived calli. After testing several media previously proposed in the literature to regenerate eggplant plantlets from explants of different origins, the results presented hereby clearly point to the use of M1 (Miyoshi 1996) to obtain the highest rate of shoot-producing organogenic calli. However, this medium alone is not sufficient to promote shoot growth. According to our results, we propose the repeated subculture (approximately on a monthly basis) of the microspore-derived calli in M1, and then the repeated subculture of the excised shoots in basal MS medium (M11) to promote the development of new normal-appearing organs and the rooting of shoots, and therefore the formation of entire plantlets. As shown in Table 1, this procedure allowed us to obtain 7.6 plants every 100 cultivated calli, which represents a remarkable improvement (~4x) compared with the 2% obtained in the only previous reference of entire plant regeneration from eggplant microspore-derived calli (Miyoshi 1996). We also showed that media supplemented with GA_3, and in particular M13, produced more elongated shoots, ready for rooting, than any other media. However, the inability of this medium to promote root growth, and the increased time and expenses associated to the use of an additional step with M13, make us to discourage its use. Finally, we obtained a high frequency of DH

individuals (69.3%), which is also higher than those previously reported for eggplant DHs coming from both anther and microspore culture. To increase this frequency, the genome of the 5.7% haploids might be duplicated with colchicine or oryzalin (Dhooghe et al. 2011). However, ~70% of DHs seems a percentage good enough for most breeding programs, and makes us propose to discard the addition of a step for genome doubling of the haploid individuals, which can just be disposed of.

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References

Asif M, Eudes F, Randhawa H, Amundsen E, Spaner D (2014) Phytosulfokine alpha enhances microspore embryogenesis in both triticale and wheat. Plant Cell Tissue Organ Cult 116: 125-130

Borgato L, Conicella C, Pisani F, Furini A (2007) Production and characterization of arboreous and fertile *Solanum melongena* plus *Solanum marginatum* somatic hybrid plants. Planta 226: 961-969

Castillo AM, Nielsen NH, Jensen A, Vallés MP (2014) Effects of n-butanol on barley microspore embryogenesis. Plant Cell Tissue Organ Cult 117: 411-418

Corral-Martínez P, Seguí-Simarro JM (2012) Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.). Euphytica 187: 47-61

Corral-Martínez P, Seguí-Simarro JM (2014) Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins. Euphytica 195: 369-382

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissues *in vitro*. Plant Cell Tissue Organ Cult 104: 359-373

Dumas de Vaulx R, Chambonnet D (1982) Culture *in vitro* d'anthères d'aubergine (*Solanum melongena* L.): stimulation de la production de plantes au moyen de traitements à 35ºC associés à de faibles teneurs en substances de croissance. Agronomie 2: 983-988

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8: 377-424

Eshaghi ZC, Abdollahi MR, Moosavi SS, Deljou A, Seguí-Simarro JM (2015) Induction of androgenesis and production of haploid embryos in anther cultures of borage (*Borago officinalis* L.). Plant Cell Tissue Organ Cult: 1-9. doi: 10.1007/s11240-015-0768-5

Franklin G, Sheeba CJ, Sita GL (2004) Regeneration of eggplant (*Solanum melongena* L.) from root explants. In Vitro Cellular & Developmental Biology-Plant 40: 188-191

Gisbert C, Prohens J, Nuez F (2006) Efficient regeneration in two potential new crops for subtropical climates, the scarlet (*Solanum aethiopicum*) and gboma (*S. macrocarpon*) eggplants. New Zeal J Crop Hort Sci 34: 55-62

Kaur M, Dhatt AS, Sandhu JS, Gosal SS (2011) In vitro plant regeneration in brinjal from cultured seedling explants. Indian Journal of Horticulture 68: 61-65

Kim M, Park E-J, An D, Lee Y (2013) High-quality embryo production and plant regeneration using a two-step culture system in isolated microspore cultures of hot pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult 112: 191-201

Miyoshi K (1996) Callus induction and plantlet formation through culture of isolated microspores of eggplant (*Solanum melongena* L). Plant Cell Rep 15: 391-395

Mohinuddin AKM, Chowdhury MKU, Abdullah Zaliha C, Napis S (1997) Influence of silver nitrate (ethylene inhibitor) on cucumber *in vitro* shoot regeneration. Plant Cell Tissue Organ Cult 51: 75-78

Moshkov IE, Novikova GV, Hall MA, George EF (2008) Plant Growth Regulators III: Gibberellins, Ethylene, Abscisic Acid, their Analogues and Inhibitors; Miscellaneous Compounds. In EF George, MA Hall, GJ De Klerk, eds), Plant Propagation by Tissue Culture, Ed 3 Vol 1. Springer, Dordrecht, The Netherlands

Parra-Vega V, Renau-Morata B, Sifres A, Seguí-Simarro JM (2013) Stress treatments and in vitro culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult 112: 353-360

Rotino GL (1996) Haploidy in eggplant. In SM Jain, SK Sopory, RE Veilleux, eds), In vitro haploid production in higher plants, Vol 3. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 115-141

Salas P, Prohens J, Seguí-Simarro JM (2011) Evaluation of androgenic competence through anther culture in common eggplant and related species. Euphytica 182: 261–274

Seguí-Simarro JM (2015) Androgenesis in solanaceae. In MA Germanà, M Lambardi, eds), In vitro embryogenesis. Springer Science + Business Media, The Netherlands, p In Press

Seguí-Simarro JM, Corral-Martínez P, Parra-Vega V, González-García B (2011) Androgenesis in recalcitrant solanaceous crops. Plant Cell Rep 30: 765-778

Seguí-Simarro JM, Nuez F (2006) Androgenesis induction from tomato anther cultures: callus characterization. Acta Hort 725: 855-861

Sgamma T, Thomas B, Muleo R (2015) Ethylene inhibitor silver nitrate enhances regeneration and genetic transformation of *Prunus avium* (L.) cv Stella. Plant Cell Tissue Organ Cult 120: 79-88

Shivaraj G, Rao S (2011) Rapid and efficient plant regeneration of eggplant (*Solanum melongena* L.) from cotyledonary leaf explants. Indian J Biotechnol 10: 125-129

Tuberosa R, Sanghineti MC, Conti S (1987) Anther culture of eggplant *Solanum melongena* L. lines and hybrids. Genética Agrária 41: 267-274

Veen H, van de Geijn S (1978) Mobility and Ionic Form of Silver as Related to Longevity of Cut Carnations. Planta 140: 93-96

Xing Y, Yu Y, Luo X, Zhang JN, Zhao B, Guo YD (2010) High efficiency organogenesis and analysis of genetic stability of the regenerants in *Solanum melongena*. Biol Plant 54: 231-236

Zhang P, Phansiri S, Puonti-Kaerlas J (2001) Improvement of cassava shoot organogenesis by the use of silver nitrate *in vitro*. Plant Cell Tissue Organ Cult 67: 47-54

Tables

Table 1: Performance of the M11, M12, M13 and M14 media after 6 months expressed as the number of shoots presenting spontaneous rooting, ready for transference to rooting medium (RM), acclimated from RM, and total acclimated (spontaneous rooting + acclimated from RM). Numbers between brackets express percentages.

Medium	Shoots	Calli	Spontaneous rooting	Transferred to RM	Acclimated from RM	Total acclimated	Yield (plants/100 calli)
M11	150	501	19 (12.7)	27 (18%)	19 (70.4)	38 (25.3)	**7.6%**
M12	128	428	1 (0.8)	14 (10.9)	10 (71.4)	11 (8.6)	**2.6%**
M13	153	511	2 (1.3)	40 (26.1)	29 (72.5)	31 (20.3)	**6.1%**
M14	124	415	2 (1.6)	19 (15.3)	13 (68.4)	15 (12.1)	**3.6%**
Total	555	1855	24	100	71	95	

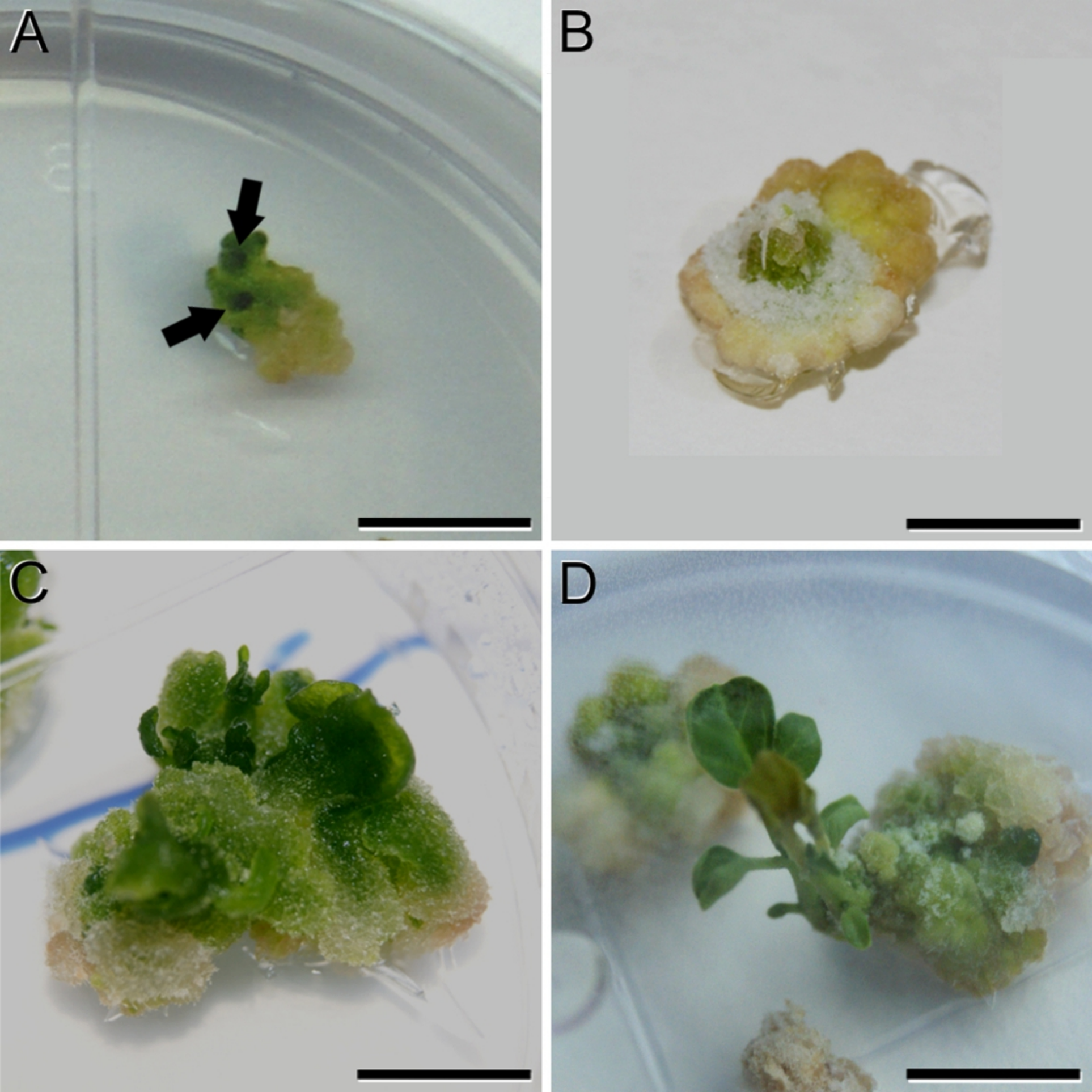
Figure legends

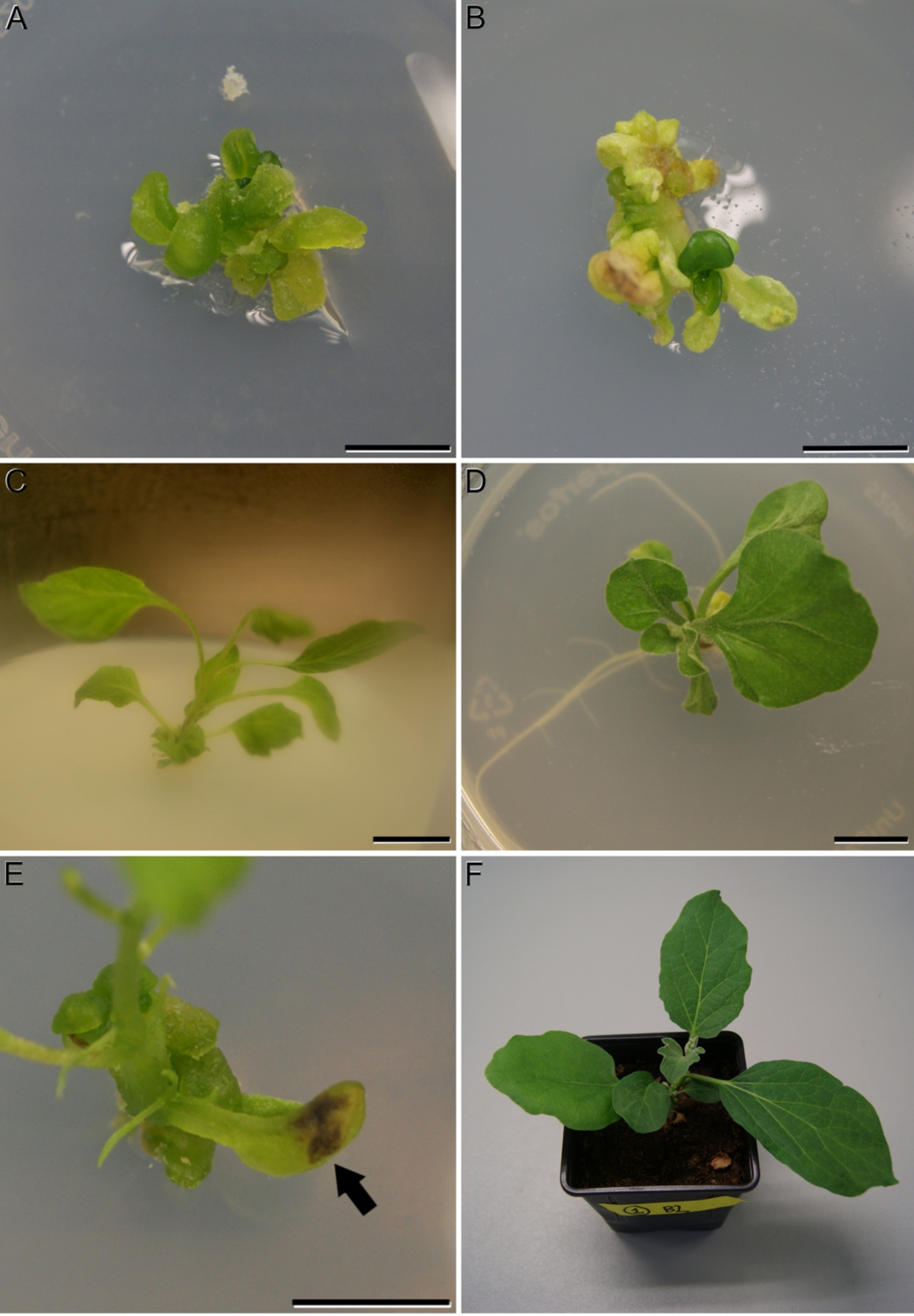
Figure 1: Evolution of calli in shoot induction media. A: Organogenic callus after two weeks in shoot induction media. Note the formation of shoot primordia (arrows) on the callus surface. B: Non-organogenic callus after 5 weeks in shoot induction media. C: organogenic callus after 5 weeks in shoot induction media. D: Shoot emerging from a callus. Bars: 1 cm.

Figure 2: Different types of shoots formed after 30 days in elongation media. A: type 1, no shoot growth and formation of swollen, hyperhydrated organs with no stem growth; B: type 2, formation of new, no hyperhydrated organs, but no stem elongation; C: type 3, formation of new, no hyperhydrated organs and stem elongation; D: type 4, root formation in addition to all the features of type 3. E: presence of necrotic lesions in shoots cultured in $AgNO_3$-containing M14 medium. F: Fully regenerated and acclimated, microspore-derived eggplant DH plant. Bars: 1 cm.

Figure 3: Percentages of the different shoot types observed after 30 days in four elongation media: M11 (A), M12 (B), M13 (C) and M14 (D). For each elongation medium the shoots produced are classified in five types: type 0 (dead shoots), type 1 (no growth, or formation of swollen, hyperhydrated organs with no stem growth), type 2 (formation of new, no hyperhydrated organs, but no stem elongation), type 3 (formation of new, no hyperhydrated organs and stem elongation) and type 4 (root formation in addition to all type 3 features. The percentages above the brackets correspond to the sum of the shoot types forming new, normal-appearing organs (types 2+3+4).

Figure 4: A: Frequencies of the elongated and rooted shoots observed after 6 months in the elongation media used. B: Time needed by shoots cultured in different elongation media to be ready for transference to rooting medium. See text for further details.


A
B
C
D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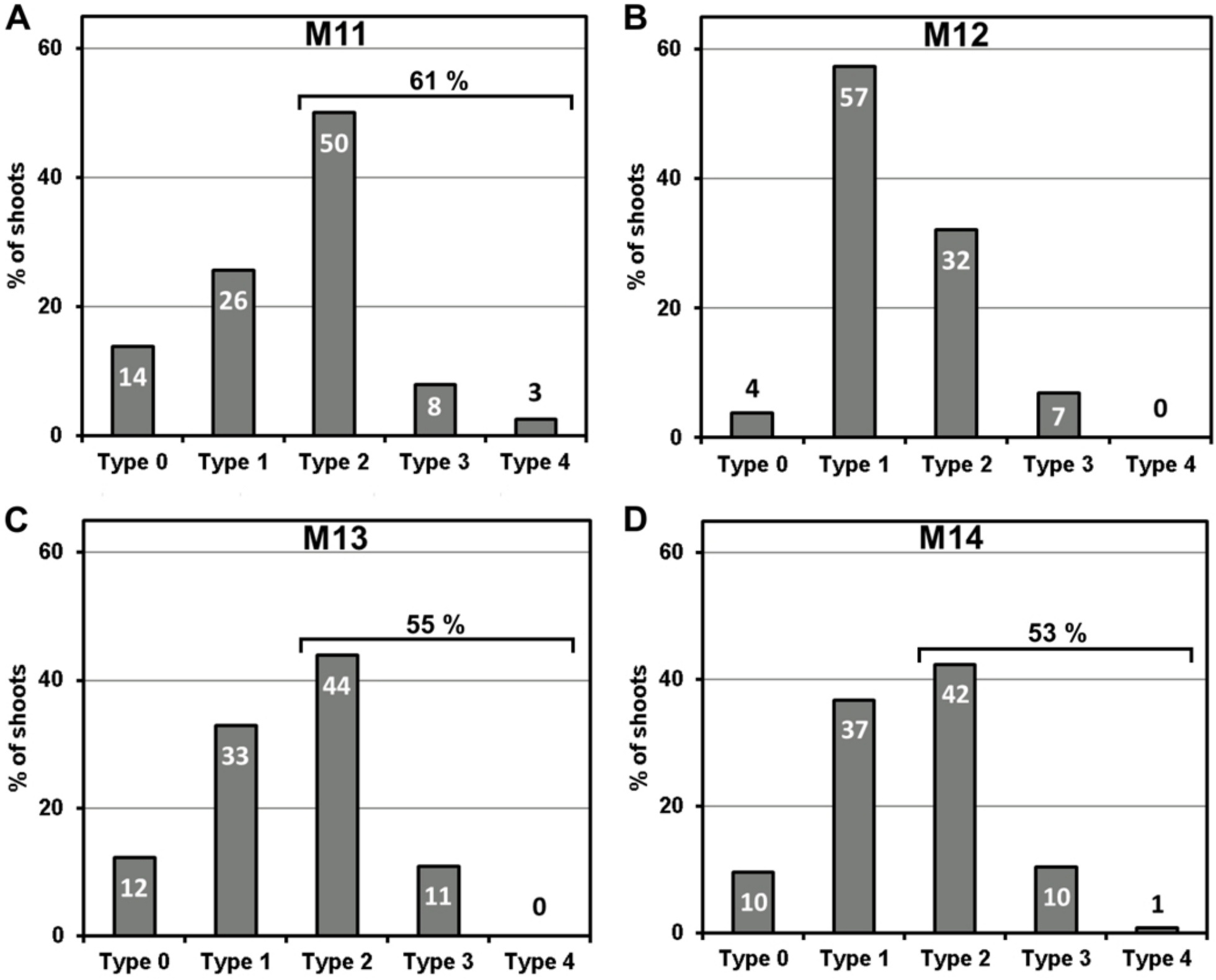
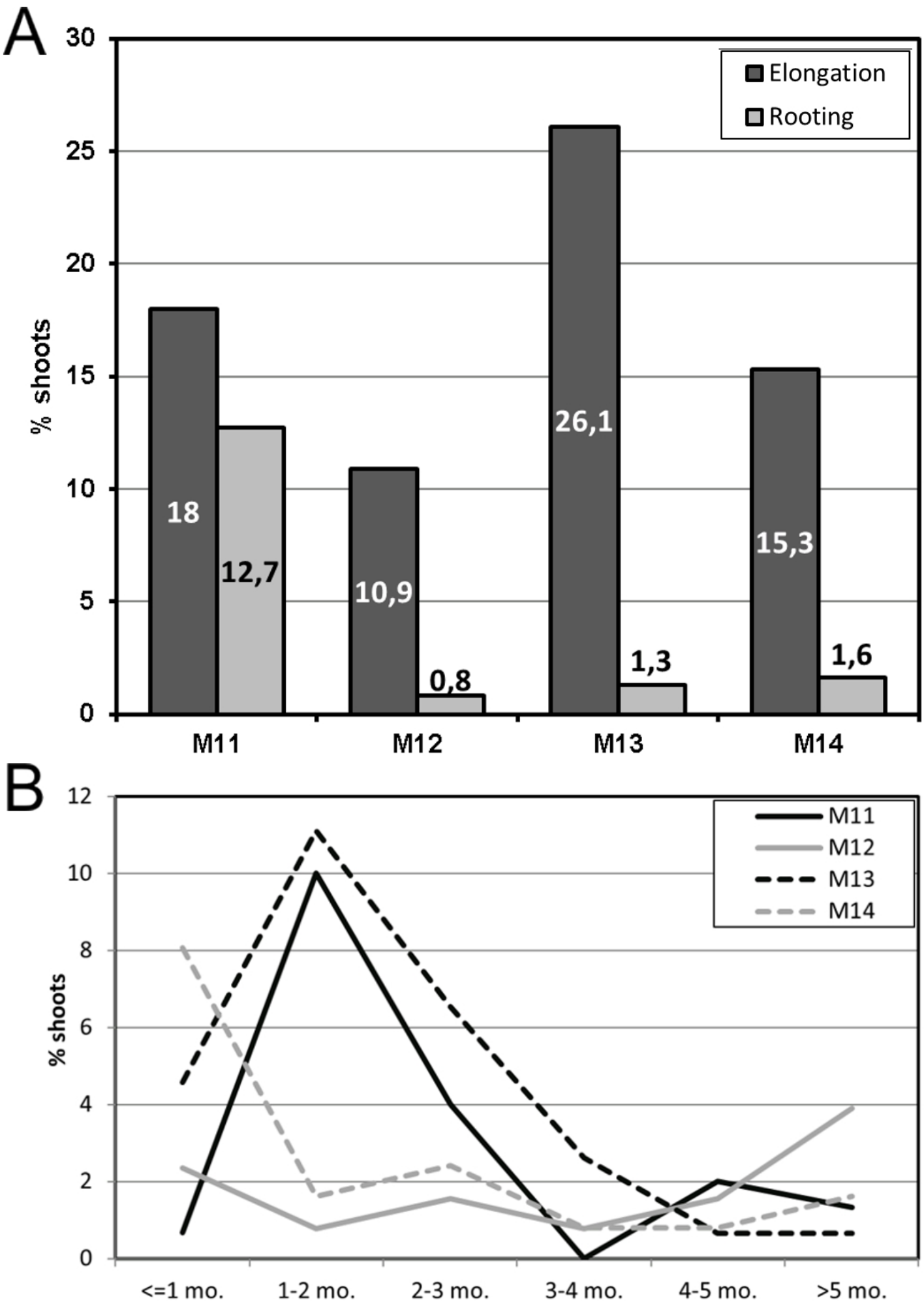

A
M11
61 %
% of shoots
60
40
20
0
14
26
50
8
3
Type 0
Type 1
Type 2
Type 3
Type 4
B
M12
57
32
4
7
0
C
M13
55 %
44
33
12
11
0
D
M14
53 %
42
37
10
10
1


A
30
25
20
15
10
5
0
% shoots
Elongation
Rooting
18
12,7
10,9
0,8
26,1
1,3
15,3
1,6
M11
M12
M13
M14
B
12
10
8
6
4
2
0
% shoots
M11
M12
M13
M14
<=1 mo.
1-2 mo.
2-3 mo.
3-4 mo.
4-5 mo.
>5 mo.