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Evaluation of the androgenic competence of 66 wild Turkish *Vaccaria hispanica* (Mill.) Rauschert genotypes through microspore culture

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Abstract

One of the most remarkable natural plants is *Vaccaria hispanica* (Mill.) Rauschert, which has a high economic, medicinal and industrial potential due to its valuable compounds. However, it is mostly an underused plant worldwide. To implement doubled haploid technology in plant breeding programs and exploit its potential, first knowing the particulars of the species is necessary. This study was aimed to explore the androgenic potential of different wild Turkish *V. hispanica* genotypes collected from different Turkish regions, as a starting point to identify bottlenecks to solve in future studies. As to induction of microspore embryogenesis, nearly all of them responded, with efficiencies reaching 300 embryos/100 buds in some cases. However, we also found three main bottlenecks. First, the presence of foam-producing saponins in flowers prevented an efficient isolation of microspores. Second, embryos showed a reduced ability to germinate. Third, a dense network of hairy roots prevented the formation of a true, fully functional root system. Together, these drawbacks led to the production of very few DH plants. The presence of rhizogenic endophytes may be the cause of most of these problems, which opens the door for possible solutions.

Abbreviations: DH: doubled haploid.

For the nutraceutical market, one of the most popular plants is *Vaccaria hispanica* (Mill.) Rauschert, also known as cow cockle. In a recent review, Zhou et al. (2016) listed 63 phytochemical compounds isolated from *V. hispanica* with anti-fungal, anti-inflammatory, anti-oxidant and anti-tumor activities, including triterpenoid saponins (42%), flavonoids (15%), and cyclic peptides (12%), among others. Given its economic potential and importance as a medicinal-industrial plant, the generation of breeding lines or hybrid varieties would be highly desirable. For this, the production of pure, fully homozygous lines is a valuable and convenient approach. Doubled haploid (DH) technology serves the fastest way to obtain pure lines in just a single generation, which allows for considerable savings of time, labor and economic resources (Segui-Simarro, 2010). Therefore, as the first step, it is important to screen the performance of existing genetic resources with an appropriate DH technique such as isolated microspore culture. In *V. hispanica*, there are only two studies reporting the successful production of DHs, both by isolated microspore culture (Ferrie et al., 2005; Kernan and Ferrie, 2006). Therefore, it seems necessary to increase the amount of available data in this species, studying other *V. hispanica* genotypes. In this work, we used 66 wild Turkish *V. hispanica* genotypes collected from all regions of Turkey and representing the genetic pool available in Turkey. We cultured their microspores following the currently available protocols, and found several problems specific to these materials. This work is a first step to produce DHs in these materials of high industrial value, and sheds light on the specific features of DH production in *V. hispanica*.

Seeds from 66 *V. hispanica* genotypes representing all regions of Turkey were collected and grown as previously described (Cam et al., 2018). Flower buds were divided into five groups with five replications according to their lengths ranging from 4 to 14 mm. The anthers within a flower develop asynchronously (Fig. 1A), which implies the coexistence of microspores at different developmental stages. We stained the microspore populations obtained from the different bud length groups with 4',6-diamidino-2-phenylindole (DAPI) and found that 5-9 mm was the length range where more microspores are at the vacuolated (Fig. 1B) and young pollen stages (Fig. 1C), the optimal stages most often identified for successful induction of microspore embryogenesis (Seguí-Simarro, 2010).

Next, we isolated the microspores in 2 mL conical tubes according to the method of Takahashi et al. (2011). A total of around 30-50 buds of each genotype were disinfected with 70% ethanol, then with sodium hypochlorite (5% active chlorite), and rinsed three times with sterile ddH_2O. Later, buds with 1 mL half-strength Gamborg's B5 (Gamborg et al., 1968)

isolation medium containing 13% sucrose (½B5-13 medium, pH 6) and few 4 mm sterile tungsten balls were macerated in a tissue lyser-like mixer mill at 1,200 rpm for 10 min, then centrifuged at low speed and filtered through 50 µm filters. However, upon crushing the buds and agitating, a very dense soap-like foam formed in the tubes (Fig. 1D), indicative of the abundant presence of saponins (El Aziz et al., 2019). Saponins are described to accumulate in seeds, leaves and roots (Meesapyodsuk et al., 2007). Thus, it was not expected to observe such a high saponin content in flower buds, which precluded an efficient microspore isolation. We then repeated these steps but according to the Kernan and Ferrie (2006) protocol, but using 50 mL tubes, where foam formation was less limiting (Fig. 1E) due to the higher volumes of the tube and the solutions used. Flower buds in tea strainers were disinfected and rinsed as described above, transferred to a glass beaker with 3 mL of cold ½B5-13 medium (pH 6) and crushed using a syringe piston. The suspension (30 mL) was filtered into 50 mL conical, centrifuged at 125*g* at 4°C for 3 min and resuspended in 5 mL of fresh ½B5-13 isolation medium. After three centrifugation-resuspension repeats, microspore density was adjusted with NLN culture medium (Lichter, 1982) with 15% sucrose, pH 6. Our *V. hispanica* genotypes produced a microspore yield insufficient to adjust the density to 50,000 microspores/mL (Fig. 1F, G) as described by Kernan and Ferrie (2006), so we reduced it to 25,000. Sterile plastic 60x15 mm dishes were loaded with 4 mL of microspore suspension (~100,000 microspores/plate), incubated at 32°C for 3 days and moved to 24°C for 4-6 weeks, always in darkness.

After the isolation and washing steps, we still observed in the isolated microspore suspensions the presence of small, clear and round/oval grains of few µm in size (Fig. 1F, G). Consistent with the occurrence of foam during isolation and the high starch content of this species, they might well be saponin or starch deposits. The first embryogenic microspore divisions were observed at the end of the first week (Fig. 1H), and the first embryos could be observed about a month later (Fig. 1I). The embryogenic responses of the 66 wild Turkish *V. hispanica* genotypes, expressed as average number of embryos per 100 buds, are shown in Fig. 1J. We found statistically significant differences among them. Only three of them did not produce any embryo, whereas 63 responded to microspore embryogenesis, being genotypes 42 (Fig. 2A) and 7 the best performing ones, with average yields of 300.6 and 167.7 embryos/100 buds, respectively. The average yields of the responsive genotypes ranged between 2.6 and 300.6 embryos/100 buds, for a total average of 49.3 embryos/100 buds. Similar genotype-dependent differences were reported in other *V. hispanica* materials by

Kernan and Ferrie (2006), when cultured at a density of 50,000 microspores/mL. This implies that some of the wild Turkish genotypes produced a nearly two times higher response in terms of embryo-producing microspores.

When whitish embryos became visible, they were moved to a growth chamber at 22°C and 16/8 photoperiod (50 $\mu mol.m^{-2}.s^{-1}$) for one week to turn green. Then, they were transferred to 24°C in B5 medium with 2% sucrose and 0.8% agar (pH 5.8) to germinate.

A total of 7,068 embryos from 63 genotypes were obtained, of which 2,530 (35.8%) were at the cotyledonary stage (Fig. 2B). A total of 1,065 cotyledonary embryos were transferred to germination medium, and 124 (11.64%) germinated (Fig. 2B). Non-germinating embryos were irregular, sometimes locally swollen, with a soft consistency and a creamy color (Fig. 2C). They eventually developed no shoots. Instead, they produced small, hair-like roots (hereinafter referred to as hairy roots), shorter and much thinner than regular roots, on the surface of the culture medium. They were observed in all culture stages from the beginning of embryo formation (Figs. 2C, D). Upon transference to the germination medium, they formed dense hairy roots (Fig. 2E) which, then entirely covered the medium surface (Fig. 2F). Almost half of the *in vitro* plantlets produced were unable to develop a true root system, which precluded shoot formation and eventually led them to death. Deficient rooting was also described for microspore-derived embryos of other *V. hispanica* materials (Ferrie et al. (2005). However, such a massive formation of surface hairy roots has never been reported.

In order to increase germination efficiency and embryo quality, we tested different concentrations of sucrose, glucose, half-strength Murashige and Skoog (1962; MS) medium salts, activated charcoal, silver nitrate and different PGRs (Suppl. Tables S1). However, the results in all cases were negative, not improving the previous results.

Sixty-six germinated embryos were transferred to larger vessels for further development. At this stage, we observed precocious *in vitro* flowering in one of these plants (Fig. 2G). This phenomenon could be induced by the stress of in vitro culture conditions, including the limited space, or could be indicative of a rapid *in vitro* aging tendency of the wild *V. hispanica* genotypes, since similar events have previously been observed in this and other *Vaccaria* species (Ari and Buyukalaca, 2006; our unpublished results). *In vitro* flowering may be advantageous for *in vitro* pollination and/or fertilization purposes, but it may also be indicative of a short life span of these plants after acclimatization. Thirty-one regenerated plantlets were transferred to pots with vermiculite, peat moss and perlite (1:2:1 v/v/v) and acclimatized. Twenty-eight died, most likely due to the insufficient root growth and the lack

of a true, healthy root system, and only three survived (Fig. 2H). We compared their ploidy with that of a diploid donor plant by flow cytometry (Fig. 2I). Young leaf pieces (~0.5 cm^2) were processed using the Partec CyStain UV Precise P Kit for nuclear extraction and staining as described (Camacho-Fernández et al., 2018) and then injected into a CyFlow Ploidy Analyzer (Partec GmbH, Germany). Two plants were DHs (Fig. 2J) and one was haploid (data not shown).

The main problem found in all of our wild Turkish *V. hispanica* genotypes was the massive production of hairy roots, both *in vitro* and *ex vitro* (Suppl. Figs. S1A, B), as also documented for other *V. hispanica* genotypes (Ari and Buyukalaca, 2006). To understand the cause of such a phenomenon, we checked out the effect of different germination conditions. We surface-disinfected and germinated seeds in wet filter paper (Suppl. Fig. S1C) and in culture media solidified with different agents, including agar (Suppl. Fig. S1D), Gelrite, vermiculite, peat and perlite (Suppl. Fig. S1E), and observed a massive occurrence of hairy roots in all cases. To adsorb possible rhizogenic substances from the medium, we added activated charcoal (Suppl. Fig. S1F). Again, hairy roots covered the surface. These results indicated that wild Turkish *V. hispanica* genotypes are remarkably prone to develop hairy roots irrespective of the growth conditions, genotype and explant. In other words, the cause of this behavior seemed endogenous rather than exogenous.

Among the possible internal causes, a possibility was the presence of particular phytochemicals in the explants (Thorpe and Murashige, 1968). We performed small-scale *in vitro* seed germination assays (Suppl. Tables S2) in MS medium containing 1 and 10 µM concentrations of different chemicals, including cytokinins such as 6-benzylaminopurine (BA), auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) and 1-N-naphthylphthalamic acid (NPA) and a systemic fungicide (imazalil). We also tried different light conditions including combinations of monochromatic blue, red, far-red LED light and fluorescent. However, none of these approaches was able to effectively inhibit hairy root formation without compromising healthy plant growth. The second possible scenario was the presence of endophytes. We used non-contaminated, abnormal embryos (Fig. 3A) and hairy root samples (Fig. 3B) to investigate the possible presence of fungi and bacteria. For fungus detection, embryos and hairy roots were incubated with 250 µg/mL trypan blue in lactic acid, glycerol and water (1:1:1) for 30 min and examined under a light microscope (Calis et al., 2015). The preparations confirmed the presence of blue-stained fungi, as revealed by the

occurrence of spore-like structures (Fig. 3C) and strongly stained hyphae intermingled with root hairs (Figs. 3D, E).

For bacterial identification, hairy root pieces were crushed in dH_2O and 500 µl of suspension were spread on culture dishes with 2% nutrient medium (5 g/L peptone, 3 g/L beef extract, 12 g agar-agar, 1 L dH_2O, pH 7.0) and incubated at 28°C for 48 h, after which bacterial colonies were visually evident (Fig. 3F), and dishes emitted malodor, indicative of bacterial growth. Microscopical examination revealed the presence of rod-shaped bacteria (Fig. 3G). In addition, we performed a Ryu test (Ryu, 1940; Powers, 1995) where bacterial colony samples were incubated for 5-10 s on a slide with 3% KOH, which revealed the Gram-negative nature of these colonies. These findings pointed to endophytes as the main cause of excessive rhizogenesis. Indeed, it is known that endophytes can alter the root system architecture by disrupting polar auxin transport (Wang et al., 2016). Even more interestingly, endophytes are responsible for high saponin contents in other species such as *Panax ginseng* (Yan et al., 2019) and *Panax notoginseng* (Jin et al., 2017; Han et al., 2020). All this considered, both the massive rhizogenesis and the high saponin contents of *V. hispanica* genotypes studied could be due to the presence of endophytes.

In summary, this study, aimed to identify bottlenecks to solve in order to produce DH in wild Turkish *V. hispanica* genotypes, revealed that most genotypes (63 out to 66) responded to induction of microspore embryogenesis, having some of them remarkably high embryo yields. However, embryo germination was unsuccessful. This should be the first bottleneck to focus on. The high foam-producing saponin content in flowers may also be preventing an efficient microspore isolation, thereby increasing the amount of inducible microspores. The last main bottleneck relates to the massive production of short hairy roots, which precludes the formation of true, fully functional root systems. The next step should be designing strategies to reduce the presence of endophytic microorganisms to prevent this phenomenon. In conclusion, *V. hispanica* microspore embryogenesis appears promising but has still a large potential for improvement.

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Authors Contribution

EA conceived and designed the research, conducted experiments, analyzed the data and wrote the manuscript. IGD and IG collected wild plant materials. HB performed the experiments and collected the data. JMSS analyzed the data, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

All data generated or analyzed during this study are included in this article. The data are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

Consent for publication

The authors declare consent for publication.

Research involving human and/or animal participants

This research did not involve experiments with human or animal participants.

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Figure Legends

Fig. 1. Stages of microspore culture in *Vaccaria hispanica*. A: Asynchronous anther development. B: Late uninucleate microspore. C: Early binucleate pollen grain. D: Dense foam formation in a 2 mL centrifuge tube upon crushing the buds. E: Reduced foam after microspore isolation in 50 mL tubes. F, G: Microspores counted in a hemacytometer. Note the low density of microspores and the presence of small, light grains. H: Dividing, embryogenic microspore after one week of culture. I: Torpedo embryo (*arrow*) after one month of culture. J: Androgenic performance of the 66 wild Turkish *Vaccaria hispanica* genotypes studied, expressed as average number of embryos produced per 100 buds. For each of the 66 *V. hispanica* genotypes, isolated microspore cultures were repeated at least three times with a minimum of three culture dishes for each repetition, during a period of two consecutive years. Data were subjected to analysis of variance followed by the Tukey test to separate genotype means with $\alpha \leq 0.05$. The vertical bars represent the standard errors. Different letters indicate statistically significant differences. Bars: B, C: 30 μm; F-H: 50 μm; I: 2 mm.

Fig. 2. Production of DHs in wild Turkish *Vaccaria hispanica* from microspore-derived embryos. A: Microspore-derived embryos obtained from 6 weeks 'genotype 42' cultures. B: Germinating embryo with two cotyledons, a defined hypocotyl and short hairy root formation from 5 weeks culture. C: Embryos developing abnormally at 4 weeks of culture. D: Short hairy root formation in a young microspore-derived embryo developing at 3 weeks of culture. E, F: Dense short hairy root formation during embryo regeneration both in culture dishes (4 weeks old) (E) and in larger vessels (almost 1 year old) (F). G: *In vitro* flowering of a DH plantlet 9 weeks after initial culture. H: Acclimatized regenerant 10 weeks after initial culture. I, J: Flow cytometry peaks from a control diploid plant (I) and a DH plant (J). Bars: A: 1 cm; B: 2 mm; C: 5 mm; D: 250 μm; E, F: 1 cm.

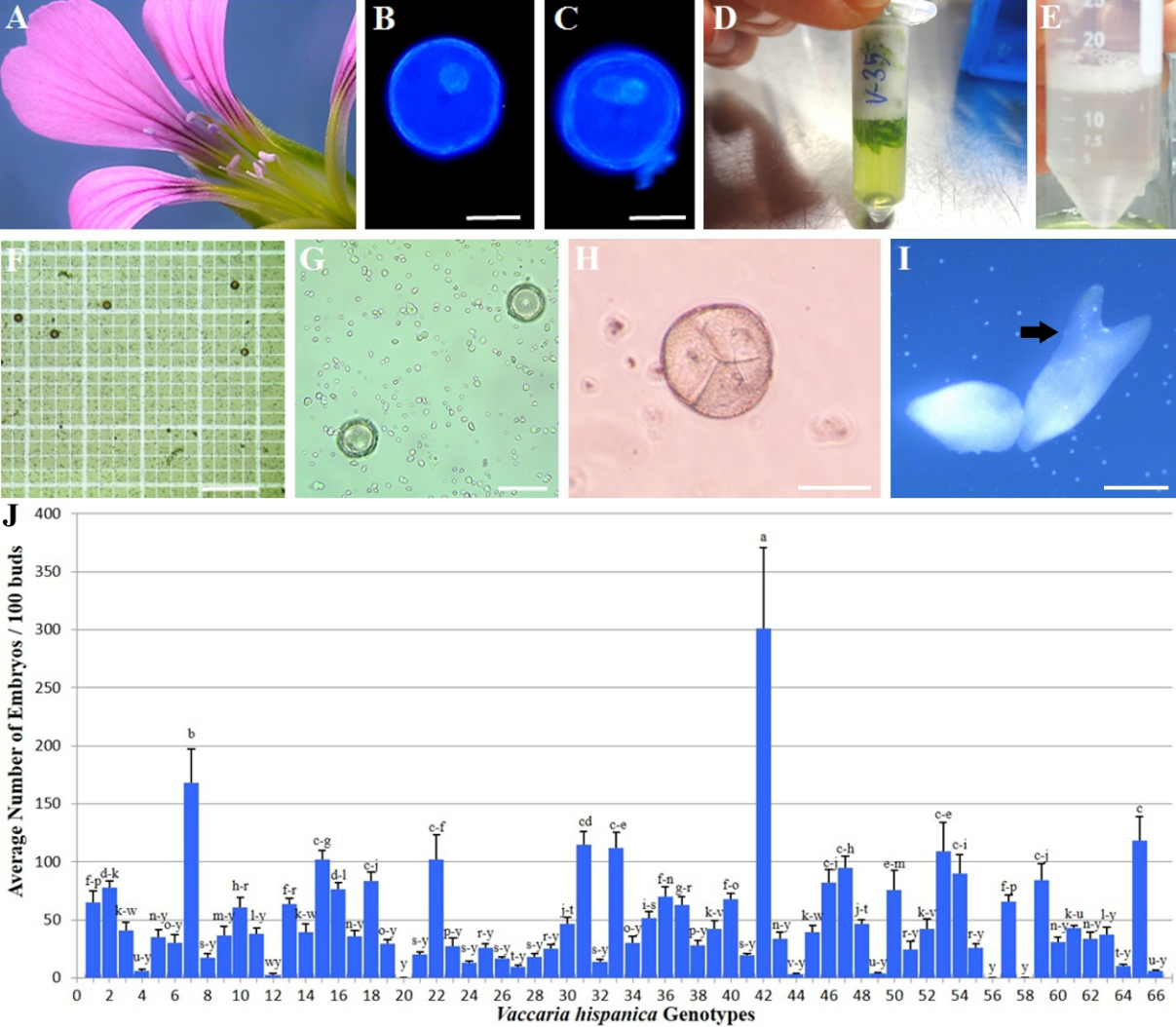
Fig. 3. Study of the presence of endophytes. A, B: Trypan blue-stained fungi observed in the surface of an abnormal microspore-derived embryo (A) and a root with abundant hairy roots (B). C-E: Spore-like (C) and hyphal-like structures (D-E) found within the hairy root network. F: Growth of bacterial colonies from hairy root samples on 2% nutrient agar medium at 28°C for 48 hours. G: Rod-shaped bacteria (*arrows*) identified in non-contaminated hairy root samples. Bars: A, B: 500 μm; C, D: 200 μm; E: 50 μm; F: 1 cm; G: 20 μm.

Supplementary Materials

Suppl. Fig. S1. Massive hairy root formation in *Vaccaria hispanica.* A, B: Hairy roots formed while keeping backup seedling trays for 3 weeks in the greenhouse to renew the planted seedlings if required. C: Hairy roots formed after 1 week seed germination on wet filter paper in culture plates. D: Hairy roots formed after 2 weeks seed germination in agar-solidified *in vitro* culture medium. E: Hairy roots formed during *in vitro* seed germination in 3-month culture media containing Gelrite (from left to right, the first three tubes), vermiculite, peat and perlite (the 4^{th}, 5^{th} and 6^{th} tubes, respectively). F: Hairy roots formed during microspore-derived embryo regeneration in 6-month culture medium containing activated charcoal.

Suppl. Table S1. Experiments of different medium modifications performed on different *V. hispanica* genotypes to increase the efficiency of isolated microspore culture and embryo germination.

Suppl. Table S2. Experiments with different chemicals added to cultures and different light sources and their effects on seed germination, hairy root formation and shoot development in small-scale *in vitro* seed germination assays in *V. hispanica* genotype 35. Root and shoot growth densities were determined according to 0-4 visual rating scale [0: none (-), 1: very little (*), 2: little (**), 3: moderate (***), and 4: abundant (****)].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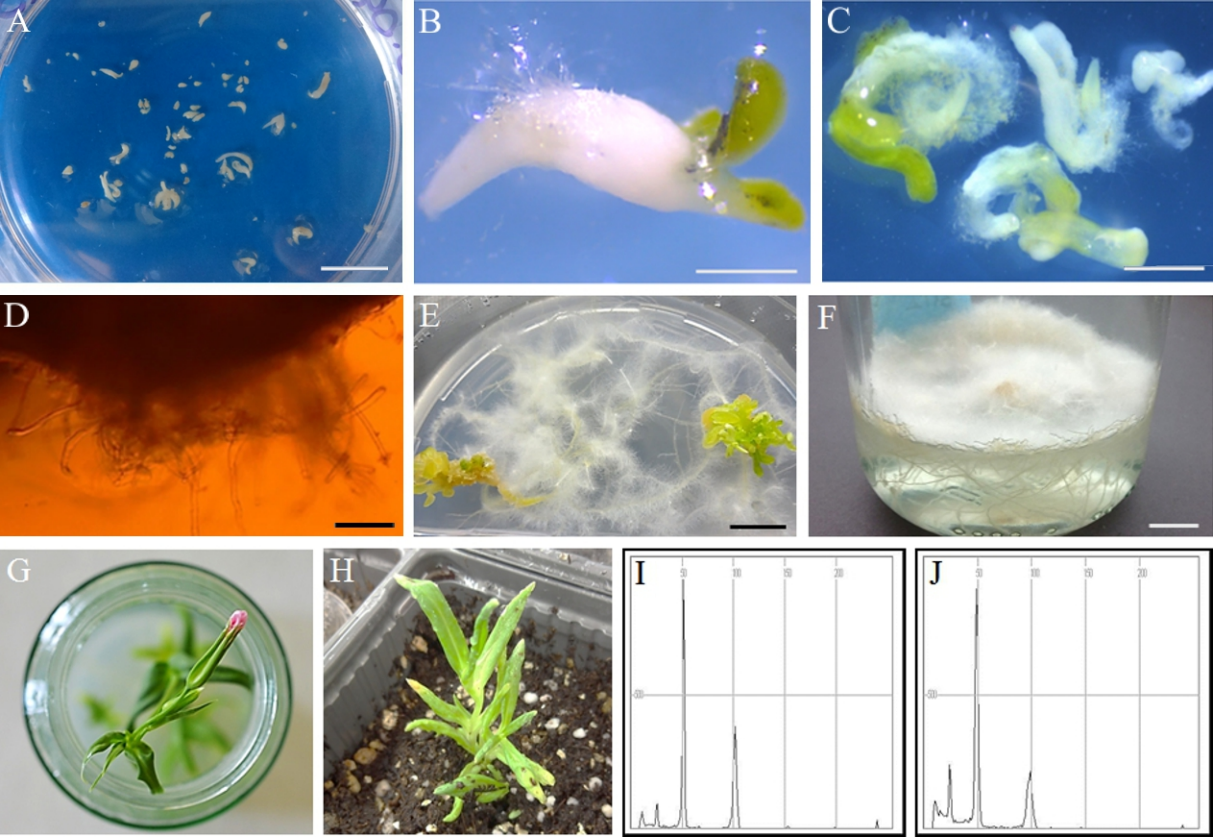
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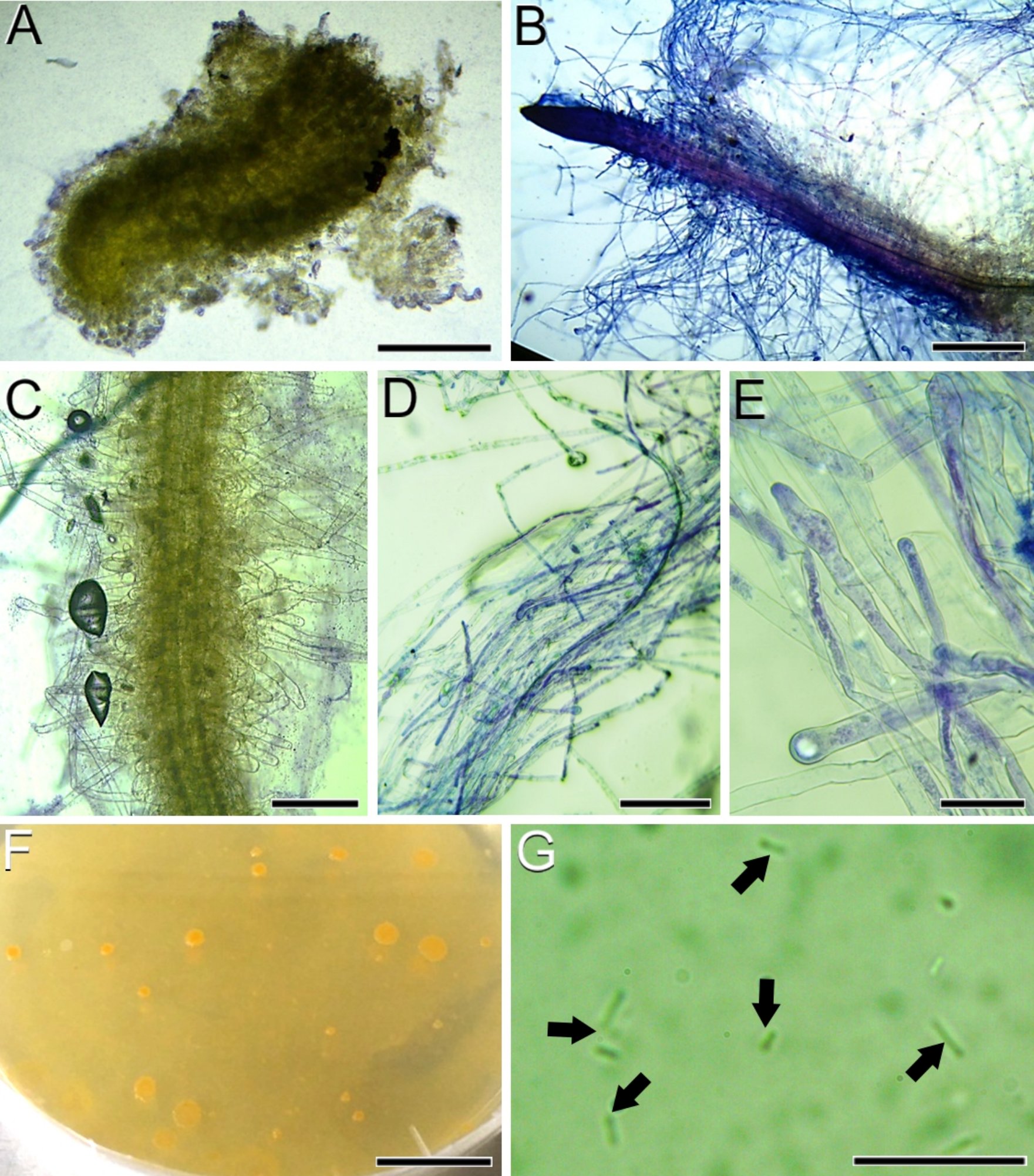
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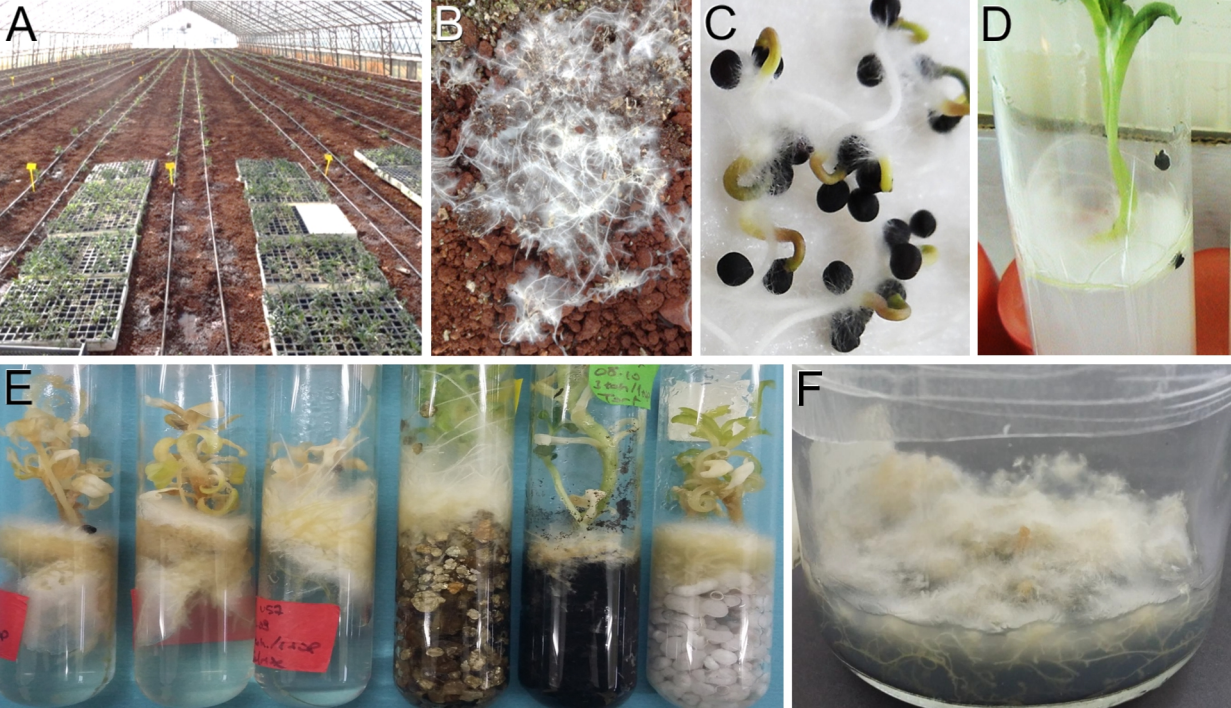
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