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Original Research

Effect of the genotype, explant source and culture medium in somatic embryogenesis and organogenesis in *Vaccaria hispanica* (Mill.) Rauschert

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Abstract

Vaccaria hispanica is an interesting species with attractive agronomic properties and a wealth of valuable bioactive compounds, potentially useful for many different purposes. Surprisingly, the number of studies focused on the development of *in vitro* tools for a rapid production of clonal populations is extremely limited. In the present study, two wild Turkish genotypes, previously characterized as high starch and saponin producers, are used to explore the possibilities of regenerating clonal plants through somatic embryogenesis and organogenesis. This work investigates the independent effects of genotype, type of explant and composition of the culture medium, and the interactions among them, in the growth and proliferation of calli from the explants, and the induction of somatic embryogenesis and organogenesis fron the callus surface. Some of the interactions were found significant to promote these processes. *V. hispanica* proved to be especially responsive for callus induction from all the explants tested. Particular explant types and combinations of plant growth regulators have been identified as especially suitable to induce the different morphogenic processes. *V. hispanica* is remarkably prone to produce adventitious roots, which may be a problem when trying to induce somatic embryogenesis or shoot organogenesis. However, this can be exploited to develop a convenient system for *in vitro* secondary metabolite production.

Keywords: Cow cockle, Endophyte, Thin adventitious root, Organogenesis, Somatic embryogenesis, *Vaccaria hispanica*.

1. Introduction

The size of the global nutraceutical market is expected to reach $722 billion (U.S.) by 2027 (Grand View Research 2020). The reason for this rapid growth is the increased interest, need and demand for natural medicinal and aromatic plants. This high economical potential is driving efforts to produce higher amounts of plant-derived metabolites and to develop new varieties with

55 superior content. For this, one of the most popular natural plants at present is *Vaccaria hispanica*
56 (Mill.) Rauschert, also known as cow cockle. It belongs to the family Caryophyllaceae and has
57 several synonyms such as *V. segetalis* (Neck) Garke, *V. pyramidata* Medik, and *Saponaria*
58 *vaccaria* L. (Zhou et al. 2016). It is an annual herbaceous plant widely distributed in Asia and
59 Europe (Sang et al. 2003), and introduced to North America (Duddu et al. 2015). Under natural
60 conditions, it grows up to 70 cm and has pink flowers opening in April-July.
61 Although used in Chinese medicine for nearly 2000 years (Zhou et al. 2016), *V. hispanica* is
62 an underutilized medicinal plant in many countries of the world, except for several local
63 ethnobotanical uses as treatments against rheumatism, tumors, menstrual disorders and for
64 enhancement of lactation (Cakilcioglu and Turkoglu 2011; Ishtiaq et al. 2021). However, its
65 attractive agronomic properties and valuable bioactive compounds have made *V. hispanica* an
66 important candidate as a new alternative medicinal and industrial crop (Willenborg and Johnson
67 2013). This plant has been studied mostly for the determination of its medicinal properties. In a
68 recent review, Zhou et al. (2016) listed 63 phytochemical compounds from *V. hispanica* having
69 anti-fungal, anti-inflammatory, anti-oxidant and anti-tumor activities. The most valuable
70 compounds are usually small-sized (0.5-1.6 μm) starch grains (60-65%) and saponins (2-4%;
71 Balsevich 2008). Saponins are a diverse group of plant defensive phytochemicals with a unique
72 biological ability to foam. This is why they have been used as surfactant and foaming agents in
73 industry. Besides, they have anticancer and anticholesterol activities, which has extended their
74 interest to the pharmaceutical sector (Güçlü-Üstündağ and Mazza 2007). In *V. hispanica*, saponin
75 typically accumulate in seeds, where the content may range from 0.64 % to 2–4% (Mazza et al.
76 1992; Balsevich 2008), and to a lesser extent in leaves, roots (Meesapyodsuk et al. 2007), and
77 even flower buds (Ari et al. 2022). Other valuable compounds include ribosome-inactivating
78 proteins (11-14%), flavonoid-type antioxidant phenolics (0.4-1%), and 0.3-1% segetalin-type
79 cyclic peptides (Balsevich 2008). In addition, new metabolites such as hevein-like peptides
80 (vaccatides; Wong et al. 2017), vitexin (Orhan et al. 2017) and hypaphorine (Chen et al. 2018)
81 have been recently reported. These substances are useful in the herbal, nutraceutical, veterinary,

82 medicinal, vaccine (as adjuvant), food, feed (as additive) and cosmetics industries (Balsevich
83 2008). Given its economic potential and importance as a medicinal-industrial plant,
84 biotechnological approaches to produce clonal plant populations in a reduced time become
85 essential to explore all the potential of this species.

86 *In vitro* culture techniques offer large-scale potential for a rapid multiplication. Somatic
87 embryogenesis and organogenesis have been widely used to produce clonal populations amenable
88 to exploit the valuable chemical compounds and secondary metabolites of medicinal and aromatic
89 plants in much higher quantities. Both morphogenic processes originate from founder, totipotent
90 stem cells characterized by a high potential for proliferation and differentiation into multiple cell
91 and tissue types (Verdeil et al. 2007). Somatic cells of different organs can transform into
92 pluripotent stem cells by various signals under different *in vitro* conditions (Verdeil et al. 2007).
93 Somatic embryogenesis is considered an extreme example of the developmental plasticity of
94 plants for survival in nature (Feher 2015). It is described as the process whereby a bipolar,
95 functional embryo forms from a non-zygotic cell, developing a vascular system independent of
96 the original tissue (Von Arnold et al. 2002). In *in vitro* conditions, four conditions are required
97 for somatic embryogenesis: potential, competence, induction and commitment. This means that
98 the individual must have the genetic potential to form embryos, and at least a few cells of the
99 explant must be competent to perceive a signal that commits them to embryogenesis (Feher 2005).
100 Another strategy for plant survival is *de novo* shoot and root organogenesis from detached organs.
101 This can also be induced *in vitro* from excised tissues or organs, provided that the right balance
102 of plant growth regulators (PGRs) is supplied by the culture medium (Chen et al. 2014). High
103 auxin/cytokinin ratios are used to induce roots, whereas low ratios generally form shoots
104 (Christianson and Warnick 1983). A number of medicinal plants respond positively to the
105 induction of these processes (Verma et al. 2011; Alonso-Herrada et al. 2016; Deepak et al. 2019).
106 In particular, several saponin-producing plants, including *Aesculus hippocastanum* (Radojevic,
107 1988), *Bacopa monnieri* (Faisal et al. 2018), *Panax ginseng* (Tang 2000) and *Sapindus trifoliatus*
108 (Asthana et al. 2011; 2017), have been successfully cloned by somatic embryogenesis, shoot

109 organogenesis or both. However, reports on clonal propagation in an important saponin-
110 producing species such as *V. hispanica* are still scarce (Koga et al. 2000; Ari and Buyukalaca
111 2006; Schmidt et al. 2007; Condie et al. 2011; Bao et al. 2016). Furthermore, to the best of our
112 knowledge, there are no studies on induction of somatic embryogenesis in this species.

113 In a previous work, 66 wild Turkish *V. hispanica* genotypes collected from all regions of
114 Turkey and representing the genetic pool available in Turkey, were analyzed within the frame of
115 a project aimed to lay the foundations for *V. hispanica* breeding in Turkey (Cam et al. 2018).
116 Seeds of these materials were analyzed for agronomical (size, diameter, weight, bulk density) and
117 chemical traits (moisture and contents in starch, protein, saponin, cyclopeptide, phenolics, fat and
118 fatty acid composition). Among the 66 genotypes, genotypes 20 and 46 were described as having
119 the highest starch (49.6 g/100 g dry seed) and total saponin (1.14 g/100 g dry seed) yields. This
120 work explores the *in vitro* morphogenic potential of these genotypes investigating the effects of
121 genotype, type of explant and composition of the *in vitro* culture medium in the induction of callus
122 proliferation, somatic embryogenesis and adventitious shoot and root organogenesis. A
123 histological analysis was performed to verify the embryogenic and organogenic origin of the *in*
124 *vitro* structures observed. A detailed statistical analysis revealed the independent effects of each
125 of the factors tested, as well as the interactions between them. Some of these interactions were
126 found remarkably relevant to increase the efficiency of the process of callus induction and the
127 occurrence of somatic embryogenesis and organogenesis from them. This work contributes to
128 widen the set of *in vitro* tools to produce clonal *V. hispanica* populations, potentially useful
129 beyond the genotypes hereby studied.
130

131 ## 2. Materials and methods
132

133 ### *2.1. Plant material*

134 Two wild *V. hispanica* genotypes from the collection described by Cam et al. (2018) were
135 used. These genotypes were chosen by their high production of starch and saponin. Seeds were

136 treated in tea strainers with 70% ethanol for 1 min, rinsed with sterile ddH_2O, surface disinfected
137 with sodium hypochlorite (20% active chlorite), and rinsed three times with sterile ddH_2O. Then,
138 seeds were germinated *in vitro* in a medium with MS salts (Murashige and Skoog 1962), Nitsch
139 and Nitsch (1969) vitamins, 4.5% sucrose and 0.7% agar (pH, 5.8). Seeds were incubated in
140 darkness for the first two days and then under a 16/8 h photoperiod at 24°C. Upon germination,
141 the seedlings were used as donors for explants (hypocotyl, cotyledon, internodes and true leaf).
142 All the chemicals used were from Duchefa (Haarlem, Netherlands) unless otherwise indicated.
143
144 *2.2. Callus induction and culture conditions*
145 Callus culture studies were carried out in two stages. In the first stage, each of the four explant
146 types were cultured in 35 different initial culture media. For the preparation of the initial culture
147 media, MS basal salts supplemented with 3% sucrose and 0.25% gelrite (pH, 5.9) were autoclaved
148 at 121 °C for 20 min. Then, different combinations (Table 1) of 2,4-dichlorophenoxyacetic acid
149 (2,4-D) and naphtalene acetic acid (NAA) as auxins, and benzyladenine (BA) and thidiazuron
150 (TDZ) as cytokinins, all dissolved in dimethylsulfoxide and filter-sterilized, were added under
151 laminar flow conditions. Finally, all media were dispensed into disposable sterile plastic dishes
152 (60×15 mm). Each of the four explant types were cultured in each initial medium in dark at 24°C.
153 Hypocotyl and internodal explants were cultured horizontally, while cotyledon and leaf explants
154 were placed with the abaxial side facing the culture medium. The callogenic response was
155 evaluated after six weeks.
156
157 *2.3. Callus subculture for induction of indirect somatic embryogenesis and organogenesis*
158 The morphogenic calli developed in each initial culture medium were equally divided into two
159 groups. One group was transferred to a subculture medium with 75% the initial concentration of
160 MS salts (¾MS) and with half of the PGR concentration of the initial medium (½PGR). The other
161 group was transferred to ¾MS medium without PGRs. The rest of medium components were left

unchanged. Six weeks after subculture, the morphogenic response in terms of somatic embryogenesis and adventitious shoot and root organogenesis was evaluated.

2.4. Histology

Two month-old calli, somatic embryos and organogenic structures at different stages were fixed in Karnovsky fixative (Karnovsky, 1965) at room temperature for 5 h, washed twice in 0.025 M cacodylate buffer + $CaCl_2.2H_2O$ solution (pH 6.9) for 30 min, dehydrated in graded ethanol series, infiltrated with liquid paraffin in the oven at 56 °C for 3 days, embedded in paraffin wax (Merck, Germany), sectioned (7 µm thickness) with an ultramicrotome (Leica RM 2125 RT, Germany), and stained with hematoxylin (O'Brien and McCully, 1981). After staining, sections were permanently mounted on glass slides and observed and photographed under a light microscope (Leica DME 750, Germany) equipped with Kameram (Argenit, Turkey) image analysis software.

2.5. Experimental design and statistical analysis

Factorial experiments were conducted according to a completely randomized design. Two groups of observation data, consisting of callus production and formation of morphogenic structures, were evaluated on an explant basis. For the first group of observations (callus induction), the effects of the factors in a 2 × 4 × 35 design (genotype × explant × initial culture medium) were analyzed and expressed as total number of callus density of callus per explant and number of morphogenic calli produced. Callus density was scored for the total calli based on a 0-5 visual rating scale, where 0 = absent, 5 = the highest. Morphogenic calli were defined as proliferative, yellowish and friable calli that end up producing any type of morphogenic structure. For the second group of observations, the effects of the factors in a 4 × 35 × 2 design (explant × initial culture medium × subculture medium) were analyzed and expressed as percentages of somatic embryos, shoots, true roots and thin adventitious roots produced after callus subculture. All the treatments were performed with four replicates and four explants in each replicate.

189 For statistical analysis, data were subjected to a three-way analysis of variance (ANOVA)
190 followed by the Least Significant Difference (LSD) test to separate means with $\alpha \leq 0.05$ using the
191 SAS-based JMP 8.0 statistical package program (SAS Institute Inc., USA). The percentage data
192 were transformed prior to analysis, but the reported means were based on the nontransformed
193 data.
194
195 **3. Results and discussion**
196
197 ***3.1. Callus formation, somatic embryogenesis and organogenesis induction***
198 Different culture media promoted the growth of different types of calli. Non-morphogenic
199 callus (Fig. 1A) were hard, compact, coriaceous and rapidly turned brown. Morphogenic calli
200 (Fig. 1B) were generally creamy-yellowish, soft, spongy and friable in texture. Light microscopy
201 sections of morphogenic calli revealed the presence of growth nodes (arrows in Fig. 1C) made by
202 small, non-vacuolated and heavily stained cells, indicative of a dense cytoplasm. These features
203 are indicative of meristematic cells. It is generally assumed that PGRs must be added to the culture
204 medium to elicit a morphogenic response since, without PGRs, explants may survive during some
205 weeks or months, but no calli are usually formed (Verma et al. 2016; Deepak et al. 2019). In *V.*
206 *hispanica*, ~95% of the explants cultured in control medium, without PGRs, produced calli (Table
207 2). However, only 7% of them transformed into morphogenic calli. This unusual feature could be
208 explained by the processing of explants prior to tissue culture. Due to the tight relationship
209 between wounding and auxin response (Da Costa et al. 2013; Xu 2018), the dissection of explants
210 from the donor plants may elicit a response to the wounding stress generated that may include the
211 accumulation of auxins and, in general, a dramatic change of the hormonal profiles of explants.
212 The new endogenous hormone profiles could well be sufficient to promote high rates of cell
213 proliferation and callus formation, but not of organogenesis. The exogenous addition of PGRs
214 would account for the transformation into morphogenic callus.

215 Six weeks after culture initiation, the calli produced were subcultured. Half of them were
216 transferred to PGR-free medium, while the other half were transferred to ½PGR medium. Some
217 calli transformed into non-morphogenic calli and did not develop further. Others developed as
218 morphogenic calli and eventually formed different structures on their surface. Some morphogenic
219 calli developed small embryogenic masses that turned into globular embryo-like structures (Fig.
220 1D). After few days, these structures transformed into heart-shaped embryos (Fig. 1F), then
221 torpedo (Fig. 1H) and finally cotyledonary somatic embryos which, in some cases, detached from
222 the callus surface (Fig. 1J).
223 Other calli developed organogenic nodules on their surface (Fig. 2A) which, after few days,
224 gave rise to organogenic structures identifiable as adventitious shoots according to their external
225 morphology (Fig. 2B) and internal anatomy (Fig. 2C). Organogenic shoots regenerated the aerial
226 parts of the plant (Figs. 2D, E). Although somatic embryos, shoots and true roots (Fig. 2F) were
227 frequently observed, the most abundant structure on the callus surface were short, highly branched
228 thin adventitious roots (Fig. 2G), very similar to those typically induced by the infection of
229 *Agrobacterium rhizogenes* and identical to those previously defined as hairy roots in *V. hispanica*
230 microspore-derived embryos (Ari et al. 2022). They most likely have the same origin, but to avoid
231 confusion with those induced by *A. rhizogenes*, they will be referred to as *thin adventitious roots*.
232 Thin adventitious roots formed soon after callus formation and rapidly covered the callus surface,
233 forming in some cases a dense network along the entire culture dish (Fig. 2H) that precluded the
234 development and the unambiguous identification of the other callus-derived structures.
235
236 ***3.2. Interactive effect of the genotype, type of explant and culture medium for callus induction***
237 Hypocotyl, cotyledon, internodal and leaf explants from genotypes 20 and 46 were cultured in
238 35 different culture media. In general, explants formed calli in all media including control. As
239 seen in Table 2, the independent effect of genotype was statistically significant in terms of
240 formation and density of total calli, and of morphogenic calli. Both genotypes produced a very
241 high number of calli, but genotype 20 showed in general the highest frequencies. It is known that

242 different plant tissues show different responses to organogenesis and embryogenesis induction
243 (Thomas et al. 2004). This is why the independent effect of the type of explant was studied. It
244 was found significant, being cotyledons the most responding explant for the three parameters
245 studied. The independent effect of the culture medium used was found to be significant too. The
246 average total callus formation in the 35 different culture media tested was 95%, of which almost
247 40% were morphogenic. Four culture media (8, 18, 28 and 32) produced callus in all the explants
248 used. These media included 2,4-D, usually at 2 mg/L. The highest callus densities and frequencies
249 of morphogenic calli were observed in media 23 and 28.
250 All the interactive effects on the three callus parameters were found to be significant (Table
251 2). The effect of the triple interaction genotype × explant × culture medium in the frequency of
252 morphogenic callus formation is shown in Table 3, which shows that each explant from each
253 genotype reacted differently to each culture medium. No common trends could be observed for
254 genotype 46. However, medium 23 promoted the highest rate of morphogenic callus formation
255 (average of 90.63%) simultaneously in all explant types of genotype 20. In general, the best media
256 to promote a morphogenic response in both genotypes were 23 and 28. Both media included 2
257 mg/L 2,4-D and 0.5-1 mg/L TDZ.
258
259 ***3.3. Effect of the genotype, type of explant and culture medium in embryogenesis and***
260 ***organogenesis***
261 The effect of the different genotypes, types of explant and culture media initially used for
262 callus induction was evaluated for the promotion of different morphogenic processes (Table 4).
263 The type of explant used was found important to determine the regeneration pathway, but in a
264 genotype-dependent manner. Genotype 20 produced somatic embryos from all explants, with no
265 statistically significant differences among them. In contrast, differences among explants were
266 observed in genotype 46, where the best explants to produce somatic embryos were cotyledons,
267 as also described for other species (Ghazi et al. 1986; Bhansali 1990). For shoot formation,
268 genotype 46 showed no significant differences among explants, as opposed to genotype 20, where

269 the highest frequency of shoot formation was found in internodal explants, as also reported for
270 other *V. hispanica* backgrounds (Schmidt et al. 2007). For root formation, both genotypes showed
271 explant-specific significant differences, and in both cases, the best source of true roots was leaf
272 explants. For thin adventitious roots, the most productive explants were hypocotyls, with
273 frequencies much higher that those of true roots, shoots or embryos. However, in this case all
274 other explants produced thin adventitious roots, also with frequencies similar or even higher than
275 those of the other structures.

276 The effects of the culture medium initially used to induce calli (Table 4) were found
277 statistically significant for all morphogenic processes and for both genotypes, except for shoot
278 formation in genotype 46. The medium yielding the highest frequency of embryo formation
279 (6.3%) for both genotypes was medium 3 (with 2 mg/L 2,4-D). This medium was not the best
280 exclusively in terms of callus production, but considering together the frequencies of callus
281 production and embryogenesis induction, medium 3 is proposed as the best to induce somatic
282 embryogenesis in the studied *V. hispanica* genotypes. For shoot formation, the highest values
283 were obtained with the control medium without PGRs in genotype 20 (10.9%) and with medium
284 10 (with 2 mg/L NAA + 0.5 mg/L BA) in genotype 46 (1.6%). True root formation occurred in
285 control medium for both genotypes, although the highest rates were obtained with media 5 and 4
286 for genotypes 20 and 46, respectively. Both media included NAA (2 and 1 mg/L, respectively).
287 However, the highest rate of thin adventitious root formation was produced in media 2 and 3 for
288 genotypes 20 and 46, respectively. Both media included 2,4-D (1 and 2 mg/L, respectively). These
289 results clearly demonstrated that in *V. hispanica,* NAA promotes true root formation, whereas
290 2,4-D induces the development of abnormal roots.

291 Subsequently, the independent effect of the subculture medium was evaluated. The PGR-free
292 medium produced significantly more normal roots in genotype 46 and more thin adventitious
293 roots in both genotypes (Table 4). According to the literature, long exposure of explants to auxin
294 may cause low frequency of callus formation, poor callus growth and loss of regeneration capacity
295 of the callus, which may obviously have an impact in organogenesis (Klimaszewska and Keller

1985; Slesak et al. 2005). Also, it may also have detrimental effects in somatic embryogenesis (Anzidei et al. 2000; Zheng and Konzak 1999). It is known that for a proper development of the somatic embryo, the endogenous levels of IAA must decrease to allow the establishment of the polar auxin gradient (Michalczuk et al. 1992; Jimenez 2005) and the polar transport of auxin, which is essential for cell polarity, the establishment of bilateral symmetry, and early stage embryo development (Liu et al. 1993). Instead, when 2,4-D is used as auxin, continuous culture in such medium increases the endogenous levels of auxin (Michalczuk et al. 1992; Minocha and Minocha 1995; Feher 2005). For this reason, exogenous auxin is generally removed or lowered after the initial culture. However, in this case, the use of ½PGR medium for subculture could be considered to reduce, if needed, the amount of thin adventitious roots, since the effect on other organogenic processes is almost negligible.

3.4. Effect of the interactions among explant type, initial culture medium and subculture medium in embryogenesis and organogenesis

The triple interaction explant × initial culture medium × subculture medium was significant for all morphogenic processes in both genotypes except for shoot formation in genotype 46 (Table 4). For simplicity, only the most relevant combinations are mentioned. The highest embryo formation (25%) was obtained with hypocotyl explants from genotype 20 first cultured in medium 3 and then in PGR-free subculture medium, with cotyledon explants from genotype 46 cultured in medium 2 or 12 and then in PGR-free subculture medium, or cultured in medium 3 and in ½PGR medium. Leaf explants of genotype 46 also formed 25% embryos in medium 3 + ½PGR subculture medium. All these media have in common the use of 1-2 mg/L 2,4-D, with or without cytokinin. In other species such as carnation, similar medium combinations with 2,4-D and subculture in PGR-free medium produced somatic embryos from internodal (Frey et al. 1992) and petal explants (Casas et al. 2010). Thus, a direct relationship can be deduced between the use of 2,4-D and the induction of somatic embryogenesis in general, concluding that in *V. hispanica*, the most relevant factor to induce somatic embryogenesis would be the use of 1-2 mg/L 2,4-D in the

induction medium. The type of explant, the presence of cytokinins in the induction medium, or the presence of hormones in the subculture medium would be less relevant.

The highest shoot formation (75%) was obtained from internodal explants of genotype 20, cultured first and then subcultured in PGR-free medium. This result suggests that the endogenous PGR content of these explants is high enough to form shoots with no exogenous PGRs. In genotype 46, the highest shoot formation (12.5%) was obtained from leaf explants cultured in medium 10 (with 2 mg/L NAA + 0.5 mg/L BA) + subculture medium with ½PGR. Equivalent results were obtained by Schmidt et al. (2007) with the same explants of other *V. hispanica* varieties cultured *in vitro* with very similar media. This makes us conclude that the combination of the type of explant used and the PGR composition of the culture medium is determinant for *in vitro* shoot formation.

As for true root formation, the best results (100%) were observed with the combination of medium 5 (with 2 mg/L NAA) and subculture in PGR-free medium. In genotype 46, this combination produced 100% rooting in hypocotyl and cotyledon explants, and 62.5% and 25% in leaf and internodal explants, respectively. In genotype 20, the rooting percentages were 87.5% and 50% for cotyledon and leaf explants, respectively. Considering both genotypes together, the best explant was the leaf, in agreement with previous studies (Koga et al. 2000; Schmidt et al. 2007; Condie et al. 2011; Bao et al. 2016). It is also noticeable that true root formation took place at percentages ranging from 12.5% to 75% in hypocotyl and leaf explants of both genotypes first cultured and then subcultured in PGR-free medium. Once again, it seemed that the endogenous PGR content of *V. hispanica* allows for morphogenesis (rooting in this case) with no need for exogenous PGR addition.

3.5. Biotechnological possibilities of in vitro thin adventitious root formation

Thin adventitious root formation occurred at a very high frequency, sometimes higher than true rooting, in all explant types of both genotypes, with up to eight different initial media, and with both subculture media. In some cases, thin adventitious roots even covered the entire culture

dish (Fig. 1j). The highest percentages of thin adventitious root formation (100%) were observed in initial media containing TDZ combined with 2,4-D or NAA. TDZ is known to alter the endogenous auxin and cytokinin metabolism and levels (Hutchison et al. 1996; Murthy et al. 1998), Thus, it seems reasonable to assume that the use of TDZ would increase thin adventitious root development in *V. hispanica* by causing an imbalance in endogenous hormone levels. The massive occurrence of thin adventitious roots inhibited other morphogenic processes such as somatic embryogenesis or shoot formation, since they were barely observed in explants with massive thin adventitious roots. This was also observed in other *V. hispanica* backgrounds (Ari and Buyukalaca 2006) and in microspore-derived embryos (Ari et al. 2022) produced from the *V. hispanica* genotypes used in the present study. In the latter work, it was proposed that the presence of endophytic microorganisms are the cause of the massive rooting observed. Endophytes are microorganisms that live in plant tissues without harming hosts (Yao et al. 2021), but they can alter root system architecture by disrupting polar auxin transport (Wang et al. 2016). Thus, endophytes are proposed as the main cause of thin adventitious root formation in organogenic calli of the *V. hispanica* materials used here, which are the same as those used by Ari et al. (2022).

As seen, thin adventitious root formation appears as an apparently undesirable and common problem for the induction of different *in vitro* morphogenic processes in *V. hispanica*. However, this feature might be advantageous for the *in vitro* production of highly valuable secondary metabolites. *V. hispanica* is a species rich in many different secondary metabolites, including saponins, which are also synthesized in the roots (Meesapyodsuk et al. 2007; Schmidt et al. 2007). The presence of endophytes could also be exploited, as they are currently seen as a new outstanding source of novel genes, proteins, natural biochemical compounds, secondary metabolites and bioactive antimicrobial natural products for medicine, industrial process and agriculture (Lima et al. 2005; Gehlot et al. 2015. Thus, the ability of *V. hispanica* to produce massive amounts of thin adventitious roots *in vitro* could be exploited as a convenient system for secondary metabolite production without the need for inducing the *in vitro* production of thin

adventitious roots, by transformation with *Agrobacterium rhizogenes* for example, as needed for other species.

4. Conclusions

The morphogenic ability of two *V. hispanica* genotypes previously selected by their high production of starch and saponin has been explored. These genotypes proved to be extremely regenerative in terms of callus induction, a first step to promote indirect somatic embryogenesis and organogenesis. This makes this species very suitable for *in vitro* culture. Callus induction and rhizogenesis was possible in all types of explants with high efficiencies. Cotyledon and leaf explants proved in general better for somatic embryogenesis whereas internodal and hypocotyl explants were better for shoot organogenesis.

For induction of morphogenic callus, the best PGR combination among those tested included 2 mg/L 2,4-D and 0.5-1 mg/L TDZ, in line with the results in other species such as *Cayratia japonica* (Zhou et al. 1994) or *Paphiopedilum* orchids (Lin et al. 2000). For somatic embryo formation, the medium with 2 mg/L 2,4-D was the most promising as also reported for carnation (Frey et al. 1992). Considering also the results of callus induction, this would be the most favorable PGR composition to induce callus proliferation and to produce somatic embryos. Culture and subculture in PGR-free medium was the most efficient way to produce organogenic shoots in one genotype, whereas in the other, the best results were obtained with NAA and BA. Similarly, true root formation occurred in both genotypes in PGR-free medium, although there were NAA-containing media more efficient for each genotype. Thus, the genotype seems to play a key role in these morphogenic pathways. Anyway, the use of PGR-free medium to induce callus growth and then shoot and true root formation avoids the occurrence of thin adventitious roots, thereby facilitating the proper development of the other organs.

Whereas true roots are in general best induced by NAA-containing media, thin adventitious roots were best induced by media containing 2,4-D and TDZ. This may indicate that the induction of true and thin adventitious roots respond to markedly different signals, which reinforces the

403 notion that the presence of endophytes in tissues is responsible for massive thin adventitious root
404 proliferation (Ari et al. 2022). Irrespective of the medium used to induce callus growth and
405 organogenesis, the PGR-free subculture medium produces more true and thin adventitious roots,
406 with no effect in somatic embryos or shoot production. Therefore, this would be the best choice
407 to produce true and thin adventitious roots for metabolite production. Instead, if the goal is, the
408 use of ½PGR subculture medium would be better to produce somatic embryos or organogenic
409 shoots while minimizing thin adventitious root formation. Together, these results provide a
410 valuable overview of the morphogenic potential of *V. hispanica*, and open the door for the
411 implementation of a thin adventitious root system in this species for *in vitro* secondary metabolite
412 production.
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426 **Authors Contribution**
427 All authors read and approved the final manuscript. EA: Conceptualization, Methodology,
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All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Consent for publication

If the research work is accepted for publication the all the authors provide their 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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613

Tables

Table 1. PGR composition of the 35 initial culture media where hypocotyl, cotyledon, internodal and leaf explants of genotypes 20 and 46 in *V. hispanica* were cultured.

PGR contents			Cytokinin						
			Control	BA			TDZ		
			0 mg/L	0.5 mg/L	1 mg/L	2 mg/L	0.5 mg/L	1 mg/L	2 mg/L
Auxin	Control	0 mg/L	1	6	11	16	21	26	31
	2.4 D	1 mg/L	2	7	12	17	22	27	32
		2 mg/L	3	8	13	18	23	28	33
	NAA	1 mg/L	4	9	14	19	24	29	34
		2 mg/L	5	10	15	20	25	30	35

Table 2. Effects of genotype, explant and culture medium on total callus formation, callus density and morphogenic callus formation in *Vaccaria hispanica.*

Source of variance					Total callus (%)		Callus density (1-5) [x]		Morphogenic callus (%)	
Genotype (G)										
Genotype 20					**95.6**	**a** [y]	2.6	b	**40.3**	**a**
Genotype 46					94.5	b	**2.7**	**a**	35.3	b
LSD 5%					1.0		0.1		2.0	
Pr > F					0.0468		<.0001		<.0001	
Explant (E)										
Hypocotyl					95.5	ab	2.6	b	43.4	b
Cotyledon					**96.3**	**a**	**3.2**	**a**	**53.4**	**a**
Internodal					93.9	c	2.5	b	31.5	c
Leaf					94.5	bc	2.4	c	22.8	d
LSD 5%					1.4		0.1		2.8	
Pr > F					0.0113		<.0001		<.0001	
Culture Medium (CM)										
Culture medium no	Auxin content 2.4-D (mg/L)	NAA (mg/L)	Cytokinin content BA (mg/L)	TDZ (mg/L)						
1	-	-	-	-	95.3	b-e	2.5	m-o	7.0	n
2	1	-	-	-	96.1	a-e	2.8	h-k	65.6	ab
3	2	-	-	-	96.9	a-d	2.4	op	66.4	ab
4	-	1		-	88.3	fg	1.8	r	19.5	m
5	-	2	-	-	94.5	c-e	1.7	r	23.4	lm
6	-	-	0.5	-	78.1	i	1.6	r	0.0	n
7	1	-	0.5	-	96.9	a-d	2.2	pq	39.8	h-j
8	2	-	0.5	-	**100.0**	**a**	2.6	k-n	43.0	f-i
9	-	1	0.5	-	98.4	a-c	2.9	f-i	44.5	e-h
10	-	2	0.5	-	95.3	b-e	2.9	f-j	50.0	d-g
11	-	-	1	-	83.6	gh	1.7	r	0.0	n
12	1	-	1	-	97.7	a-d	2.5	m-o	39.1	h-j
13	2	-	1	-	97.7	a-d	2.8	g-k	53.1	de
14	-	1	1	-	97.7	a-d	2.9	f-j	33.6	f-h
15	-	2	1	-	96.9	a-d	3.1	c-f	53.1	de
16	-	-	2	-	88.3	f-g	1.5	r	0.0	n
17	1	-	2	-	96.9	a-d	2.5	l-o	39.1	h-j
18	2	-	2	-	**100.0**	**a**	2.9	f-j	51.6	d-f
19	-	1	2	-	96.1	a-e	2.5	l-o	31.3	j-l
20	-	2	2	-	96.9	a-d	3.2	b-e	56.3	cd
21	-	-	-	0.5	92.2	ef	2.3	op	0.0	n
22	1	-	-	0.5	99.2	ab	3.1	d-g	53.1	de
23	2	-	-	0.5	98.4	a-c	3.4	ab	**73.4**	**a**
24	-	1	-	0.5	92.2	ef	2.7	i-l	41.4	g-i
25	-	2	-	0.5	95.3	b-e	3.1	c-f	37.5	h-j
26	-	-	-	1	96.9	a-d	2.4	no	0.0	n
27	1	-	-	1	96.1	a-e	3.0	e-h	51.6	d-f
28	2	-	-	1	**100.0**	**a**	**3.5**	**a**	**73.4**	**a**
29	-	1	-	1	93.8	de	2.8	f-j	28.1	k-m
30	-	2	-	1	96.1	a-e	3.2	b-e	35.9	h-k
31	-	-	-	2	81.3	hi	2.1	q	0.0	n
32	1	-	-	2	**100.0**	**a**	3.2	b-d	66.4	ab
33	2	-	-	2	99.2	ab	3.2	b-e	62.5	bc
34	-	1	-	2	96.1	a-e	2.7	j-m	37.5	h-j
35	-	2	-	2	99.2	ab	3.3	a-c	34.4	i-k
Mean					95.1		2.7		37.5	
LSD 5%					4.1		0.2		8.3	
Pr > F					<.0001		<.0001		<.0001	
G*E										
LSD 5%					2.0		0.1		4.0	
Pr > F					<.0001		<.0001		<.0001	
G*CM										
LSD 5%					5.9		0.3		11.8	
Pr > F					<.0001		<.0001		<.0001	
E*CM										
LSD 5%					8.3		0.5		16.7	
Pr > F					<.0001		<.0001		<.0001	
G*E*CM										
LSD 5%					11.7		0.7		23.6	
Pr > F					<.0001		<.0001		<.0001	

[x] Callus density data were obtained based on a 0-5 visual rating scale, where 0 = absent, 5 = the highest.
[y] Values are mean. A three-way ANOVA is used to compare means, which are separated with a Least Significant Difference (LSD) test. Means within a column followed by the different letter are significantly different at $p \leq 0.05$.

Table 3. Effect of the triple interaction genotype × explant × culture medium on the percentage of morphogenic callus formation in *Vaccaria hispanica*.

Culture medium no	Hypocotyl		Cotyledon		Internodal		Leaf	
	Genotype 20	Genotype 46	Genotype 20	Genotype 46	Genotype 20	Genotype 46	Genotype 20	Genotype 46
1 (Control)	0.0 m [x]	0.0 m	0.0 m	0.0 m	12.5 k-m	12.5 k-m	25.0 i-m	6.3 lm
2	**100.0 a**	12.5 k-m	81.3 a-d	87.5 a-c	68.8 b-f	50.0 e-i	68.8 b-f	56.3 d-h
3	**100.0 a**	25.0 i-m	68.8 b-f	62.5 c-g	87.5 a-c	37.5 g-k	81.3 a-d	68.8 b-f
4	75.0 a-e	25.0 I-m	0.0 m	6.3 lm	37.5 g-k	0.0 m	12.5 k-m	25.0 i-m
5	25.0 i-m	50.0 e-i	6.3 lm	50.0 e-i	12.5 k-m	25.0 i-m	0.0 m	18.8 j-m
6	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m
7	25.0 i-m	50.0 e-i	37.5 g-k	75.0 a-e	31.3 h-l	43.8 f-j	25.0 i-m	31.3 h-l
8	37.5 g-k	37.5 g-k	62.5 c-g	62.5 c-g	31.3 h-l	43.8 f-j	25.0 i-m	43.8 f-j
9	**100.0 a**	12.5 k-m	56.3 d-h	75.0 a-e	62.5 c-g	6.3 lm	0.0 m	43.8 f-j
10	**100.0 a**	12.5 k-m	75.0 a-e	**100.0 a**	37.5 g-k	6.3 lm	43.8 f-j	25.0 i-m
11	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m
12	62.5 c-g	25.0 i-m	37.5 g-k	87.5 a-c	25.0 i-m	37.5 g-k	31.3 h-l	6.3 lm
13	50.0 e-i	62.5 c-g	62.5 c-g	**100.0 a**	31.3 h-l	50.0 e-i	25.0 i-m	43.8 f-j
14	62.5 c-g	0.0 m	62.5 c-g	**100.0 a**	62.5 c-g	25.0 i-m	6.3 lm	50.0 e-i
15	**100.0 a**	12.5 k-m	75.0 a-e	**100.0 a**	75.0 a-e	6.3 lm	12.5 k-m	43.8 f-j
16	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m
17	62.5 c-g	25.0 i-m	56.3 d-h	87.5 a-c	25.0 i-m	18.8 j-m	12.5 k-m	25.0 i-m
18	75.0 a-e	50.0 e-i	56.3 d-h	75.0 a-e	56.3 d-h	50.0 e-i	25.0 i-m	25.0 i-m
19	37.5 g-k	75.0 a-e	62.5 c-g	87.5 a-c	31.3 h-l	18.8 j-m	0.0 m	12.5 k-m
20	**100.0 a**	37.5 g-k	93.8 ab	**100.0 a**	68.8 b-f	31.3 h-l	0.0 m	18.8 j-m
21	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m
22	75.0 a-e	75.0 a-e	50.0 e-i	75.0 a-e	31.3 h-l	37.5 g-k	37.5 g-k	43.8 f-j
23	**100.0 a**	87.5 a-c	**100.0 a**	62.5 c-g	87.5 a-c	75.0 a-e	75.0 a-e	0.0 m
24	75.0 a-e	50.0 e-i	62.5 c-g	87.5 a-c	43.8 f-j	12.5 k-m	0.0 m	0.0 m
25	62.5 c-g	50.0 e-i	43.8 f-j	43.8 f-j	50.0 e-i	25.0 i-m	6.3 lm	18.8 j-m
26	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m
27	**100.0 a**	62.5 c-g	68.8 b-f	75.0 a-e	31.3 h-l	12.5 k-m	37.5 g-k	25.0 i-m
28	**100.0 a**	75.0 a-e	93.8 ab	**100.0 a**	87.5 a-c	25.0 i-m	31.3 h-l	75.0 a-e
29	25.0 i-m	50.0 e-i	62.5 c-g	62.5 c-g	31.3 h-l	6.3 lm	12.5 k-m	12.5 k-m
30	50.0 e-i	75.0 a-e	37.5 g-k	37.5 g-k	31.3 h-l	37.5 g-k	0.0 m	18.8 j-m
31	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m	0.0 m
32	75.0 a-e	87.5 a-c	75.0 a-e	87.5 a-c	62.5 c-g	50.0 e-i	56.3 d-h	37.5 g-k
33	50.0 e-i	62.5 c-g	68.8 b-f	75.0 a-e	81.3 a-d	50.0 e-i	68.8 b-f	43.8 f-j
34	50.0 e-i	37.5 g-k	62.5 c-g	62.5 c-g	43.8 f-j	18.8 j-m	12.5 k-m	12.5 k-m
35	0.0 m	75.0 a-e	50.0 e-i	50.0 e-i	6.3 lm	62.5 c-g	18.8 j-m	12.5 k-m
Mean	**53.6**	37.1	47.7	**59.3**	38.4	25.0	21.4	24.1
LSD 5%	23.6							
Pr > F	<.0001 ***							

[x] Values are mean. A three-way ANOVA is used to compare means, which are separated with a Least Significant Difference (LSD) test. Means with different letters are significantly different at $p \leq 0.05$.

632 **Table 4.** Effects of the type of explant, initial medium and subculture medium on the percentages of the
633 different structures formed (embryos, shoots, true roots and thin adventitious roots).

Source of variance	Embryo formation (%)		Shoot formation (%)		True root formation (%)		Thin adventitious root formation (%)	
	Genotype 20	Genotype 46	Genotype 20	Genotype 46	Genotype 20	Genotype 46	Genotype 20	Genotype 46
Explant (E)								
Hypocotyl	0.4 a [x]	0.0 b	0.7 ab	0.0 a	5.7 ab	7.1 ab	**31.8 a**	**21.4 a**
Cotyledon	0.4 a	**1.1 a**	0.0 b	0.0 a	3.9 b	5.7 b	5.4 b	6.8 b
Internodal	0.2 a	0.2 b	**1.3 a**	0.0 a	0.2 c	1.3 c	1.4 c	1.6 c
Leaf	**0.7 a**	0.7 ab	0.0 b	**0.2 a**	**6.8 a**	**7.7 a**	5.5 b	6.6 b
LSD 5%	NS	0.7	0.5	NS	1.6	1.7	2.2	2.2
Pr > F	0.5360	0.0296	<.0001	0.3940	<.0001	<.0001	<.0001	<.0001
Initial culture medium (ICM)								
1 (Control)	0.0 b	0.0 d	**10.9 a**	0.0 a	18.8 b	37.5 c	3.1 f	0.0 g
2	1.6 b	4.7 ab	0.0 c	0.0 a	15.6 b	28.1 d	**45.3 a**	**51.6 a**
3	**6.3 a**	**6.3 a**	0.0 c	0.0 a	14.1 b	20.3 e	**25.0** bc	**53.1 a**
4	0.0 b	0.0 d	6.3 b	0.0 a	**43.8 a**	43.8 b	28.1 b	0.0 g
5	0.0 b	0.0 d	0.0 c	0.0 a	**39.1 a**	**53.1 a**	6.3 ef	1.6 g
6	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	0.0 g
7	0.0 b	0.0 d	0.0 c	0.0 a	3.1 c	3.1 f	39.1 a	21.9 b
8	1.6 b	0.0 d	0.0 c	0.0 a	3.1 c	0.0 f	15.6 d	17.2 bc
9	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	0.0 g
10	0.0 b	0.0 d	0.0 c	**1.6 a**	0.0 c	0.0 f	0.0 f	0.0 g
11	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	0.0 g
12	0.0 b	3.1 bc	0.0 c	0.0 a	1.6 c	0.0 f	6.3 ef	3.1 fg
13	1.6 b	0.0 d	0.0 c	0.0 a	0.0 c	1.6 f	14.1 d	20.3 b
14	0.0 b	0.0 d	0.0 c	0.0 a	3.1 c	0.0 f	12.5 de	18.8 bc
15	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	3.1 fg
16	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	0.0 g
17	0.0 b	0.0 d	0.0 c	0.0 a	3.1 c	0.0 f	18.8 cd	15.6 cd
18	1.6 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	14.1 d	6.3 e-g
19	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	3.1 f	6.3 ef	0.0 g
20	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	6.3 e-g
21	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	0.0 g
22	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	18.8 cd	3.1 fg
23	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	6.3 e-g
24	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	9.4 d-f
25	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	12.5 de	9.4 d-f
26	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	0.0 g
27	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	25.0 bc	18.8 bc
28	0.0 b	1.6 cd	0.0 c	0.0 a	0.0 c	0.0 f	12.5 de	3.1 fg
29	1.6 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	3.1 fg
30	0.0 b	1.6 cd	0.0 c	0.0 a	0.0 c	0.0 f	14.1 d	9.4 d-f
31	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	0.0 f	0.0 g
32	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	12.5 de	12.5 c-e
33	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	6.3 e-g
34	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	6.3 ef	12.5 c-e
35	0.0 b	0.0 d	0.0 c	0.0 a	0.0 c	0.0 f	12.5 de	6.3 e-g
Mean	0.4	0.5	0.5	0.0	4.2	5.5	11.0	9.1
LSD 5%	2.0	2.1	1.5	NS	4.6	5.0	6.4	5.5
Pr > F	0.0001	<.0001	<.0001	0.4692	<.0001	<.0001	<.0001	<.0001
Subculture medium (SCM)								
Without PGR	0.5 a	0.5 a	0.5 a	0.0 a	4.1 a	6.3 a	13.0 a	10.6 a
With ½ PGR	0.4 a	0.5 a	0.5 a	0.1 a	4.2 a	4.6 b	9.0 b	7.6 b
LSD 5%	NS	NS	NS	NS	NS	1.2	1.5	1.6
Pr > F	0.7349	0.7518	0.6548	0.3209	0.8861	0.0085	<.0001	0.0007
E*ICM								
LSD 5%	NS	4.1	3.0	NS	9.2	9.9	12.7	13.0
Pr > F	0.7873	0.0020	<.0001	0.4831	<.0001	<.0001	<.0001	<.0001
E*SCM								
LSD 5%	NS	NS	0.7	NS	2.2	2.4	NS	NS
Pr > F	0.2603	0.3233	<.0001	0.3940	0.0412	0.0319	0.0230	0.6822
ICM*SCM								
LSD 5%	NS	2.9	2.1	NS	NS	7.0	9.0	9.2
Pr > F	0.3752	<.0001	<.0001	0.4692	0.0571	<.0001	<.0001	<.0001
E*ICM*SCM								
LSD 5%	5.5	5.9	4.2	NS	13.0	14.1	18.0	18.4
Pr > F	0.0152	0.0007	<.0001	0.4831	<.0001	0.0131	<.0001	<.0001

[x] Values are mean. A separate three-way ANOVA is used for each genotype to compare means, which are separated with a Least Significant Difference (LSD) test. Means within a column followed by the different letter are significantly different at $p \leq 0.05$.

634

635 **Figure Legends:**

636

637 **Fig. 1.** Induction of callus formation and somatic embryogenesis in *Vaccaria hispanica*. **A:** Non-
638 morphogenic calli formed in a hypocotyl explant. **B:** Morphogenic callus from a leaf explant. **C:**
639 Histological section of a morphogenic callus where some growth nodes (arrows), defined by the
640 presence of small, non vacuolated, meristematic-like cells, can be observed. **D:** Morphogenic
641 callus from a leaf explant with globular embryo-like formations (arrows). **E:** Histological section
642 of a morphogenic callus showing the internal anatomy of globular embryo-like formations. **F:**
643 Heart-shaped somatic embryo. **G:** Histological section of a heart-shaped somatic embryo
644 emerging from a callus. **H:** Torpedo-like somatic embryo. **I:** Histological section of a torpedo-
645 like somatic embryo. Note the presence of a differentiated procambium (arrow). Cotyledons are
646 not shown as they are in a different sectional plane. **J:** Cotyledonary embryo. **K:** Histological
647 section of a cotyledonary embryo. Bars: A, B: 10 mm; C: 500 μm; D: 5 mm; E: 100 μm; F: 1 mm;
648 G: 100 μm; H: 2 mm; I: 100 μm; J: 2 mm; K: 200 μm.

649

650 **Fig. 2.** Induction of organogenesis in *Vaccaria hispanica*. **A:** Induction of abundant shoots
651 (arrows) on the surface of a callus from a cotyledon explant. **B:** Detail of an organogenic shoot.
652 **C:** Histological section of a morphogenic callus showing the internal anatomy of an organogenic
653 shoot. **D:** Shoot regeneration in subculture medium. **E:** Detail of a regenerated shoot from a
654 hypocotyl explant. **F:** Growth of true roots (arrows) and thin adventitious roots on a callus from
655 a cotyledon explant. Excessive growth of thin adventitious roots **(G)** rapidly extends over the
656 callus surface and eventually covers the entire culture plate **(H)**, precluding the growth of the
657 other structures. Bars: A: 5 mm; B, C: 200 μm; D: 10 mm; E: 5 mm; F: 5 mm; G: 2 mm; H: 1 cm.

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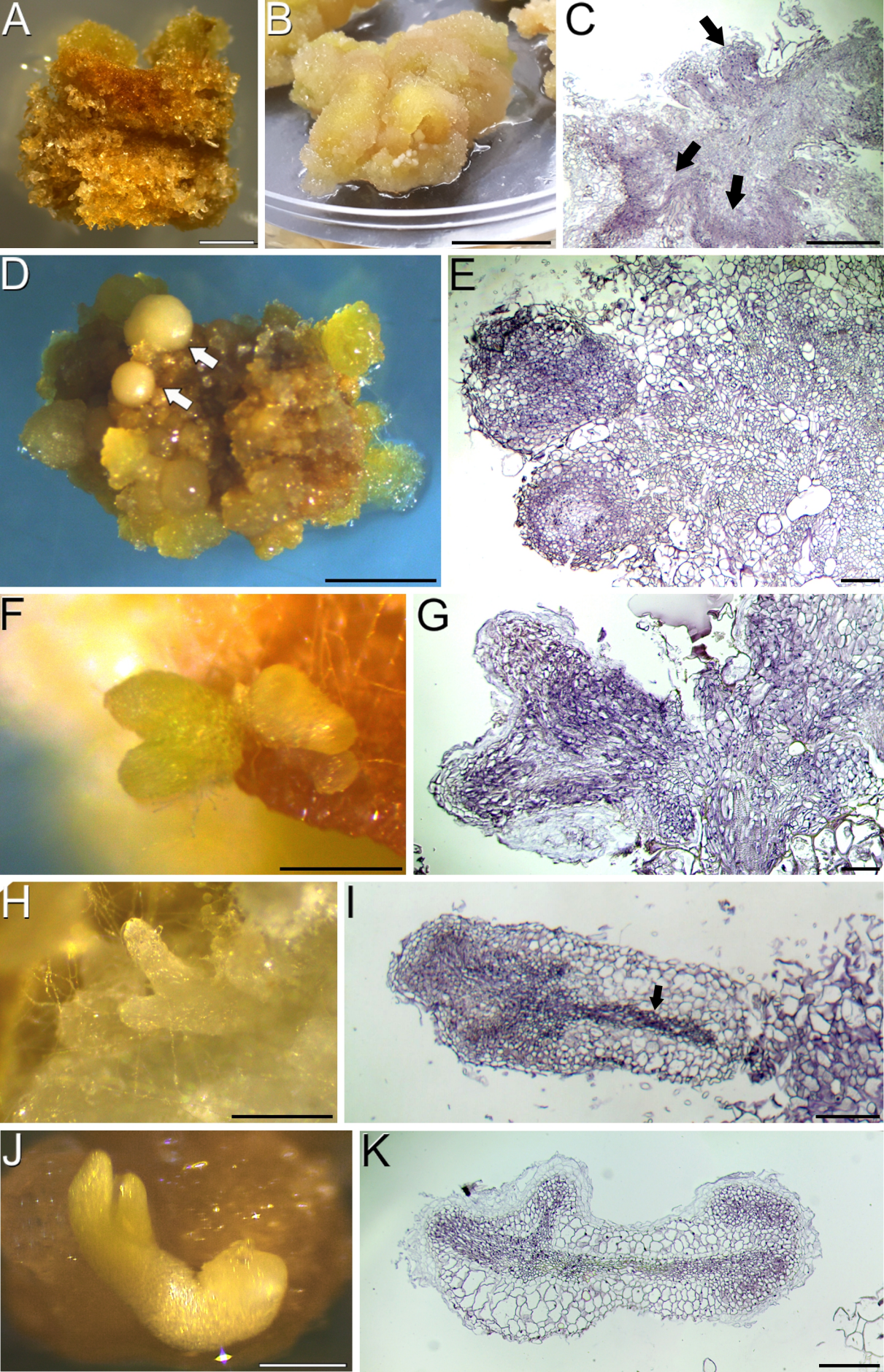
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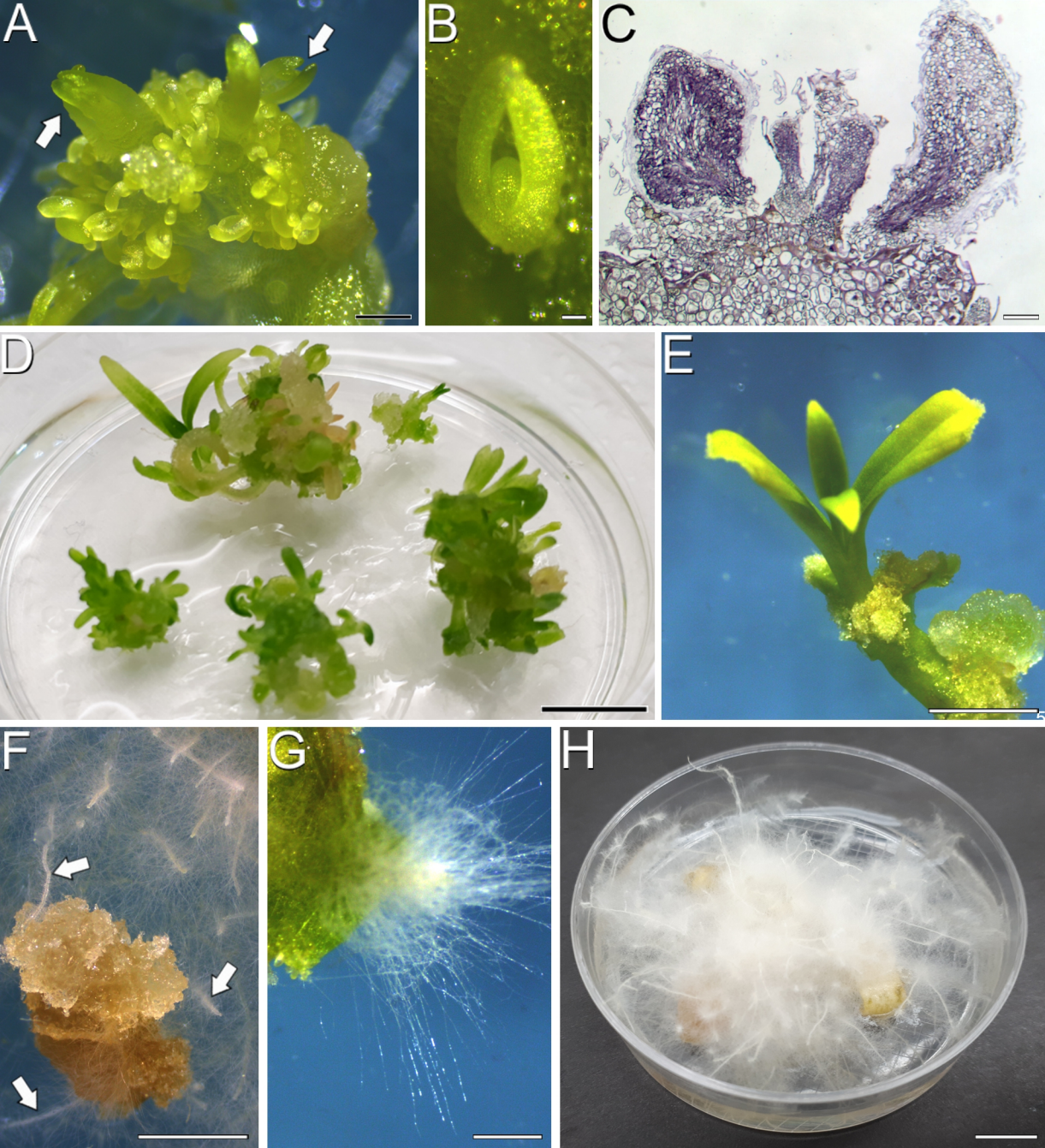
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