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ORIGINAL ARTICLE

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 thin 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.

Key Message

Vaccaria hispanica has proven to be responsive to callus formation, somatic embryogenesis and organogenesis. Also, it is prone to producing thin adventitious roots important for in vitro secondary metabolite production.

Keywords Cow cockle · Endophyte · Organogenesis · Somatic embryogenesis · Thin adventitious root · *Vaccaria hispanica*

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 superior content. For this, one of the most popular natural plants at present is *Vaccaria hispanica* (Mill.) Rauschert, also known as cow cockle. It belongs to the family Caryophyllaceae and has several synonyms such as *V. segetalis* (Neck) Garke, *V. pyramidata* Medik, and *Saponaria vaccaria* L. (Zhou et al. 2016). It is an annual herbaceous plant widely distributed in Asia and Europe (Sang et al. 2003), and introduced to North America (Duddu et al. 2015). Under natural conditions, it grows up to 70 cm and has pink flowers opening in April–July.

Although used in Chinese medicine for nearly 2000 years (Zhou et al. 2016), *V. hispanica* is an underutilized medicinal plant in many countries of the world, except for several local ethnobotanical uses as treatments against rheumatism, tumors, menstrual disorders and for enhancement

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of lactation (Cakilcioglu et al. 2011; Ishtiaq et al. 2021). However, its attractive agronomic properties and valuable bioactive compounds have made *V. hispanica* an important candidate as a new alternative medicinal and industrial crop (Willenborg and Johnson 2013). This plant has been studied mostly for the determination of its medicinal properties. In a recent review, Zhou et al. (2016) listed 63 phytochemical compounds from *V. hispanica* having anti-fungal, anti-inflammatory, anti-oxidant and anti-tumor activities. The most valuable compounds are usually small-sized (0.5–1.6 µm) starch grains (60–65%) and saponins (2–4%; Balsevich 2008). Saponins are a diverse group of plant defensive phytochemicals with a unique biological ability to foam. This is why they have been used as surfactant and foaming agents in industry. Besides, they have anticancer and anticholesterol activities, which has extended their interest to the pharmaceutical sector (Güçlü-Üstündağ and Mazza 2007). In *V. hispanica*, saponin typically accumulate in seeds, where the content may range from 0.64 to 2–4% (Mazza et al. 1992; Balsevich 2008), and to a lesser extent in leaves, roots (Meesapyodsuk et al. 2007), and even flower buds (Ari et al. 2022). Other valuable compounds include ribosome-inactivating proteins (11–14%), flavonoid-type antioxidant phenolics (0.4–1%), and 0.3–1% segetalin-type cyclic peptides (Balsevich 2008). In addition, new metabolites such as hevein-like peptides (vaccatides; Wong et al. 2017), vitexin (Orhan et al. 2017) and hypaphorine (Chen et al. 2018) have been recently reported. These substances are useful in the herbal, nutraceutical, veterinary, medicinal, vaccine (as adjuvant), food, feed (as additive) and cosmetics industries (Balsevich 2008). Given its economic potential and importance as a medicinal-industrial plant, biotechnological approaches to produce clonal plant populations in a reduced time become essential to explore all the potential of this species.

In vitro culture techniques offer large-scale potential for a rapid multiplication. Somatic embryogenesis and organogenesis have been widely used to produce clonal populations amenable to exploit the valuable chemical compounds and secondary metabolites of medicinal and aromatic plants in much higher quantities. Both morphogenic processes originate from founder, totipotent stem cells characterized by a high potential for proliferation and differentiation into multiple cell and tissue types (Verdeil et al. 2007). Somatic cells of different organs can transform into pluripotent stem cells by various signals under different in vitro conditions (Verdeil et al. 2007). Somatic embryogenesis is considered an extreme example of the developmental plasticity of plants for survival in nature (Feher 2015). It is described as the process whereby a bipolar, functional embryo forms from a non-zygotic cell, developing a vascular system independent of the original tissue (Von Arnold et al. 2002). In in vitro conditions, four conditions are required for somatic embryogenesis: potential, competence, induction and commitment. This means that the individual must have the genetic potential to form embryos, and at least a few cells of the explant must be competent to perceive a signal that commits them to embryogenesis (Feher 2005). Another strategy for plant survival is de novo shoot and root organogenesis from detached organs. This can also be induced in vitro from excised tissues or organs, provided that the right balance of plant growth regulators (PGRs) is supplied by the culture medium (Chen et al. 2014). High auxin/cytokinin ratios are used to induce roots, whereas low ratios generally form shoots (Christianson and Warnick 1983). A number of medicinal plants respond positively to the induction of these processes (Verma et al. 2011; Alonso-Herrada et al. 2016; Deepak et al. 2019). In particular, several saponin-producing plants, including *Aesculus hippocastanum* (Radojevic 1988), *Bacopa monnieri* (Faisal et al. 2018), *Panax ginseng* (Tang 2000) and *Sapindus trifoliatus* (Asthana et al. 2011; 2017), have been successfully cloned by somatic embryogenesis, shoot organogenesis or both. However, reports on clonal propagation in an important saponin-producing species such as *V. hispanica* are still scarce (Koga et al. 2000; Ari and Buyukalaca 2006; Schmidt et al. 2007; Condie et al. 2011; Bao et al. 2016). Furthermore, to the best of our knowledge, there are no studies on induction of somatic embryogenesis in this species.

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

Materials and methods

Plant material

Two wild *V. hispanica* genotypes from the collection described by Cam et al. (2018) were used. These genotypes were chosen by their high production of starch and saponin. Seeds were treated in tea strainers with 70% ethanol for 1 min, rinsed with sterile ddH_2O, surface disinfected with sodium hypochlorite (20% active chlorite), and rinsed three times with sterile ddH_2O. Then, seeds were germinated in vitro in a medium with MS salts (Murashige and Skoog 1962), Nitsch and Nitsch (1969) vitamins, 4.5% sucrose and 0.7% agar (pH 5.8). Seeds were incubated in darkness for the first 2 days and then under a 16/8 h photoperiod at 24 °C. Upon germination, the seedlings were used as donors for explants (hypocotyl, cotyledon, internodes and true leaf). All the chemicals used were from Duchefa (Haarlem, Netherlands) unless otherwise indicated.

Callus induction and culture conditions

Callus culture studies were carried out in two stages. In the first stage, each of the four explant types were cultured in 35 different initial culture media. For the preparation of the initial culture media, MS basal salts supplemented with 3% sucrose and 0.25% gelrite (pH 5.9) were autoclaved at 121 °C for 20 min. Then, different combinations (Table 1) of 2,4-dichlorophenoxyacetic acid (2,4-D) and naphtalene acetic acid (NAA) as auxins, and benzyladenine (BA) and thidiazuron (TDZ) as cytokinins, all dissolved in dimethylsulfoxide and filter-sterilized, were added under laminar flow conditions. Finally, all media were dispensed into disposable sterile plastic dishes (60 × 15 mm). Each of the four explant types were cultured in each initial medium in dark at 24 °C. Hypocotyl and internodal explants were cultured horizontally, while cotyledon and leaf explants were placed with the abaxial side facing the culture medium. The callogenic response was evaluated after 6 weeks.

Callus subculture for induction of indirect somatic embryogenesis and organogenesis

The morphogenic calli developed in each initial culture medium were equally divided into two groups. One group was transferred to a subculture medium with 75% the initial concentration of MS salts (¾MS) and with half of the PGR concentration of the initial medium (½PGR). The other group was transferred to ¾MS medium without PGRs. The rest of medium components were left unchanged. 6 weeks after subculture, the morphogenic response in terms of somatic embryogenesis and adventitious shoot and root organogenesis was evaluated.

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 + $CaCl_2 \cdot 2H_2O$ 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.

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

Table 1 PGR composition of the 35 initial culture media where hypocotyl, cotyledon, internodal and leaf explants of genotypes 20 and 46 in *Vaccaria 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

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.

For statistical analysis, data were subjected to a three-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) Student's *t* test to separate means with $\alpha \leq 0.05$ using the SAS-based JMP 8.0 statistical package program (SAS Institute Inc., USA). The percentage data were transformed prior to analysis, but the reported means were based on the nontransformed data.

Results and discussion

Callus formation, somatic embryogenesis and organogenesis induction

Different culture media promoted the growth of different types of calli. Non-morphogenic callus (Fig. 1A) were hard, compact, coriaceous and rapidly turned brown. Morphogenic calli (Fig. 1B) were generally creamy–yellowish, soft, spongy and friable in texture. Light microscopy sections of morphogenic calli revealed the presence of growth nodes (arrows in Fig. 1C) made by small, non-vacuolated and heavily stained cells, indicative of a dense cytoplasm. These features are indicative of meristematic cells. It is generally assumed that PGRs must be added to the culture medium to elicit a morphogenic response since, without PGRs, explants may survive during some weeks or months, but no calli are usually formed (Verma et al. 2016; Deepak et al. 2019). In *V. hispanica*, ~ 95% of the explants cultured in control medium, without PGRs, produced calli (Table 2). However, only 7% of them transformed into morphogenic calli. This unusual feature could be explained by the processing of explants prior to tissue culture. Due to the tight relationship between wounding and auxin response (Da Costa et al. 2013; Xu 2018), the dissection of explants from the donor plants may elicit a response to the wounding stress generated that may include the accumulation of auxins and, in general, a dramatic change of the hormonal profiles of explants. The new endogenous hormone profiles could well be sufficient to promote high rates of cell proliferation and callus formation, but not of organogenesis. The exogenous addition of PGRs would account for the transformation into morphogenic callus.

6 weeks after culture initiation, the calli produced were subcultured. Half of them were transferred to PGR-free medium, while the other half were transferred to ½PGR medium. Some calli transformed into non-morphogenic calli and did not develop further. Others developed as morphogenic calli and eventually formed different structures on their surface. Some morphogenic calli developed small embryogenic masses that turned into globular embryo-like structures (Fig. 1D, E). After few days, these structures transformed into heart-shaped embryos (Fig. 1F, G), then torpedo (Fig. 1H, I) and finally cotyledonary somatic embryos which, in some cases, detached from the callus surface (Fig. 1J, K).

Other calli developed organogenic nodules on their surface (Fig. 2A) which, after few days, gave rise to organogenic structures identifiable as adventitious shoots according to their external morphology (Fig. 2B) and internal anatomy (Fig. 2C). Organogenic shoots regenerated the aerial parts of the plant (Fig. 2D, E). Although somatic embryos, shoots and true roots (Fig. 2F) were frequently observed, the most abundant structure on the callus surface were short, highly branched thin adventitious roots (Fig. 2G), very similar to those typically induced by the infection of *Agrobacterium rhizogenes* and identical to those previously defined as hairy roots in *V. hispanica* microspore-derived embryos (Ari et al. 2022). They most likely have the same origin, but to avoid confusion with those induced by *A. rhizogenes*, they will be referred to as *thin adventitious roots*. Thin adventitious roots formed soon after callus formation and rapidly covered the callus surface, forming in some cases a dense network along the entire culture dish (Fig. 2H) that precluded the development and the unambiguous identification of the other callus-derived structures.

Interactive effect of the genotype, type of explant and culture medium for callus induction

Hypocotyl, cotyledon, internodal and leaf explants from genotypes 20 and 46 were cultured in 35 different culture media. In general, explants formed calli in all media including control. As seen in Table 2, the independent effect of genotype was statistically significant in terms of formation and density of total calli, and of morphogenic calli. Both genotypes produced a very high number of calli, but genotype 20 showed in general the highest frequencies. It is known that different plant tissues show different responses to organogenesis and embryogenesis induction (Thomas et al. 2004). This is why the independent effect of the type of explant was studied. It was found significant, being cotyledons the most responding

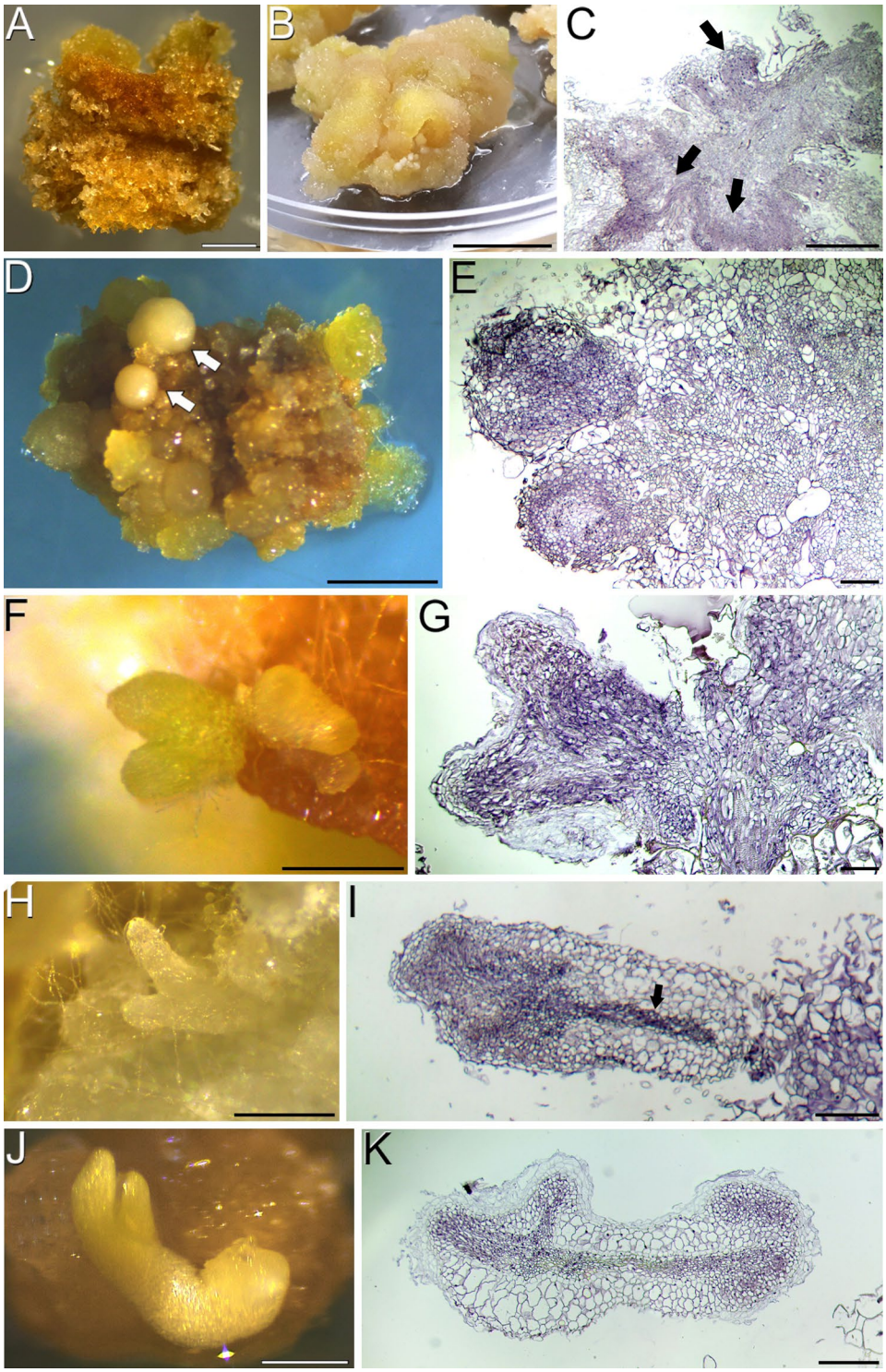

Fig. 1 Induction of callus formation and somatic embryogenesis in *Vaccaria hispanica*. **A** Non-morphogenic calli formed in a hypocotyl explant. **B** Morphogenic callus from a leaf explant. **C** Histological section of a morphogenic callus where some growth nodes (arrows), defined by the presence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

explant for the three parameters studied. The independent effect of the culture medium used was found to be significant too. The average total callus formation in the 35 different culture media tested was 95%, of which almost 40% were morphogenic. Four culture media (8, 18, 28 and 32) produced callus in all the explants used. These media included 2,4-D, usually at 2 mg/L. The highest callus densities and frequencies of morphogenic calli were observed in media 23 and 28.

All the interactive effects on the three callus parameters were found to be significant (Table 2). The effect of the triple interaction genotype × explant × culture medium in the frequency of morphogenic callus formation is shown in Table 3, which shows that each explant from each genotype

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**[ay]	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		Cytokinin content		Total callus (%)	Callus density (1–5)[x]	Morphogenic callus (%)
	2,4-D (mg/L)	NAA (mg/L)	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]	23.4l[m]
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	92.2ef	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]

Table 2 (continued)

Culture Medium (CM)							
Culture medium no	Auxin content		Cytokinin content		Total callus (%)	Callus density (1–5)[x]	Morphogenic callus (%)
	2,4-D (mg/L)	NAA (mg/L)	BA (mg/L)	TDZ (mg/L)			
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) Student's *t* test. Means within a column followed by the different letter are significantly different at $p \leq 0.05$

reacted differently to each culture medium. No common trends could be observed for genotype 46. However, medium 23 promoted the highest rate of morphogenic callus formation (average of 90.63%) simultaneously in all explant types of genotype 20. In general, the best media to promote a morphogenic response in both genotypes were 23 and 28. Both media included 2 mg/L 2,4-D and 0.5–1 mg/L TDZ.

Effect of the genotype, type of explant and culture medium in embryogenesis and organogenesis

The effect of the different genotypes, types of explant and culture media initially used for callus induction was evaluated for the promotion of different morphogenic processes (Table 4). The type of explant used was found important to determine the regeneration pathway, but in a genotype-dependent manner. Genotype 20 produced somatic embryos from all explants, with no statistically significant differences among them. In contrast, differences among explants were observed in genotype 46, where the best explants to produce somatic embryos were cotyledons, as also described for other species (Ghazi et al. 1986; Bhansali 1990). For shoot formation, genotype 46 showed no significant differences among explants, as opposed to genotype 20, where the highest frequency of shoot formation was found in internodal explants, as also reported for other *V. hispanica* backgrounds (Schmidt et al. 2007). For root formation, both genotypes showed explant-specific significant differences, and in both cases, the best source of true roots was leaf explants. For thin adventitious roots, the most productive explants were hypocotyls, with frequencies much higher that those of true roots, shoots or embryos. However, in this case all other explants produced thin adventitious roots, also with frequencies similar or even higher than those of the other structures.

The effects of the culture medium initially used to induce calli (Table 4) were found statistically significant for all

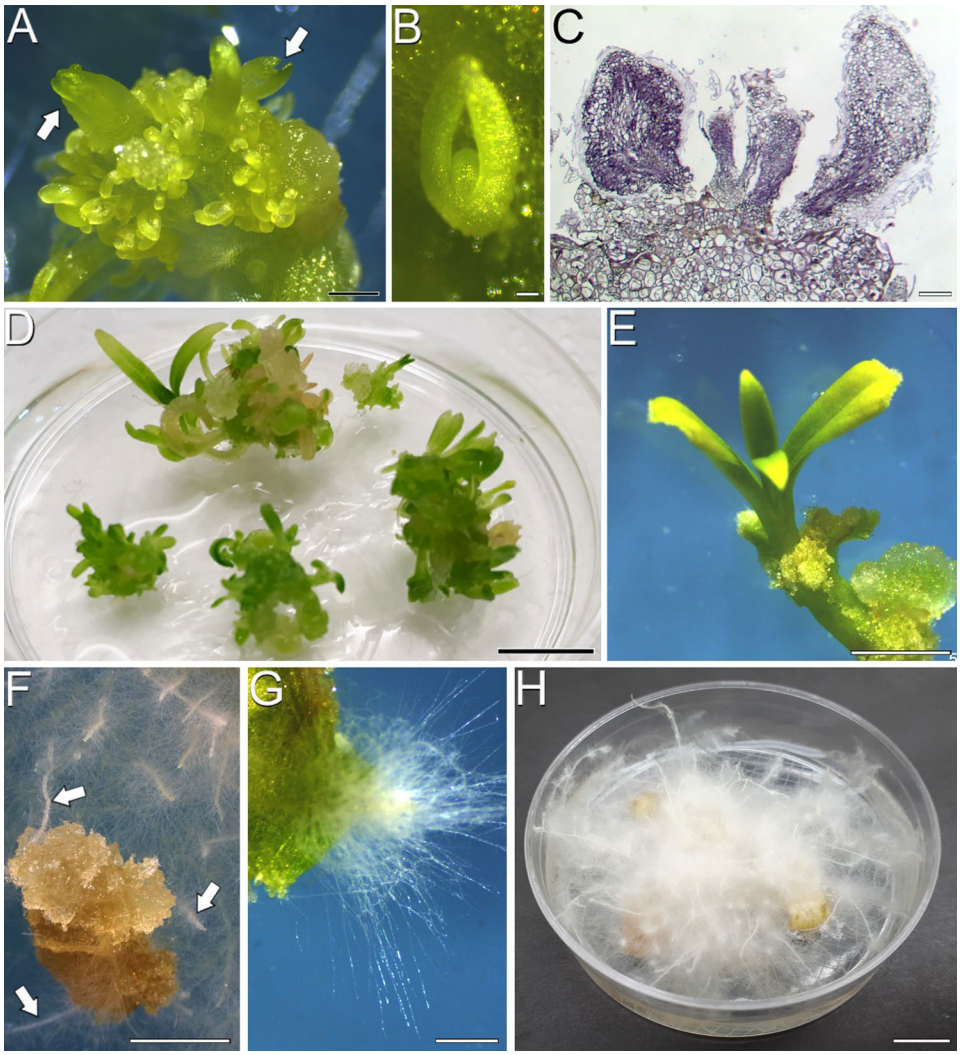

Fig. 2 Induction of organogenesis in *Vaccaria hispanica*. **A** Induction of abundant shoots (arrows) on the surface of a callus from a cotyledon explant. **B** Detail of an organogenic shoot. **C** Histological section of a morphogenic callus showing the internal anatomy of an organogenic shoot. **D** Shoot regeneration in subculture medium. **E** Detail of a regenerated shoot from a hypocotyl explant. **F** Growth of true roots (arrows) and thin adventitious roots on a callus from a cotyledon explant. Excessive growth of thin adventitious roots (**G**) rapidly extends over the callus surface and eventually covers the entire culture plate (**H**), precluding the growth of the 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

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

Subsequently, the independent effect of the subculture medium was evaluated. The PGR-free medium produced significantly more normal roots in genotype 46 and more thin adventitious roots in both genotypes (Table 4). According to the literature, long exposure of explants to auxin may cause low frequency of callus formation, poor callus growth and loss of regeneration capacity 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

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*}	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$^{l–m}$	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$^{j–m}$	0.0m	12.5$^{k–m}$
20	**100.0^{a}**	37.5$^{g–k}$	93.8ab	**100.0a**	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***							

* Values are mean. A three-way ANOVA is used to compare means, which are separated with a Least Significant Difference (LSD) Student's *t* test. Means with different letters are significantly different at $p \leq 0.05$

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,

Table 4 Effects of the type of explant, initial medium and subculture medium on the percentages of the 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^{ax}	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}	18.8b	37.5^{c}	3.1^{f}	0.0^{g}
2	1.6^{b}	4.7^{ab}	0.0^{c}	0.0^{a}	15.6b	28.1^{d}	**45.3^{a}**	**51.6^{a}**
3	**6.3^{a}**	**6.3^{a}**	0.0^{c}	0.0^{a}	14.1b	20.3e	**25.0^{bc}**	**53.1^{a}**
4	0.0^{b}	0.0^{d}	6.3^{b}	0.0^{a}	**43.8^{a}**	43.8b	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}	6.3ef	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

Table 4 (continued)

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
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) Student's *t* test. Means within a column followed by the different letter are significantly different at $p \leq 0.05$

since the effect on other organogenic processes is almost negligible.

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.

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. 2H). 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.

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 notion that the presence of endophytes in tissues is responsible for massive thin adventitious root proliferation (Ari et al. 2022).

Irrespective of the medium used to induce callus growth and organogenesis, the PGR-free subculture medium produces more true and thin adventitious roots, with no effect in somatic embryos or shoot production. Therefore, this would be the best choice to produce true and thin adventitious roots for metabolite production. Instead, if the goal is, the use of ½PGR subculture medium would be better to produce somatic embryos or organogenic shoots while minimizing thin adventitious root formation. Together, these results provide a valuable overview of the morphogenic potential of *V. hispanica*, and open the door for the implementation of a thin adventitious root system in this species for in vitro secondary metabolite production.

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Data availability All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest The authors declare no conflict of interest.

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Research involving human and/or animal participants This research did not involve experiments with human or animal participants.

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