Note

IN VITRO CULTURE FROM MATURE SEEDS OF *PASSIFLORA* SPECIES

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ABSTRACT: The genus *Passiflora* comprises hundred species, mainly native of the South American tropics and rainforests, which are grouped into 21 subgenera. Some species are widely studied for their economic importance and are chiefly cultivated for production of fruit juice. To obtain a continuous source of material for a screening of secondary metabolites, zygotic embryo culture was attempted for 62 *Passiflora* species, starting from seeds mainly collected in the wild. Twenty nine of these species produced calli, which had very different growth rates. Plants were successfully regenerated from calli of 13 different species. For 25 of the responsive species this is the first report of *in vitro* culture.
Key words: *Passiflora* regeneration, embryo culture, endosperm culture, ethnobotanical species, plant acclimatization

REGENERAÇÃO E CULTURA *IN VITRO* DE ESPÉCIES DE *PASSIFLORA*

RESUMO: O gênero *Passiflora* compõe centenas de espécies, a maioria de origem dos trópicos e das florestas da América do Sul, as quais são agrupadas em 21 subgêneros. Algumas espécies foram intensamente estudadas por sua importância econômica e são cultivadas principalmente para a produção de suco de fruta. Cultura de 29 espécies de *Passiflora* foram obtidos a partir de embriões zigóticos e de culturas de endosperma. Foram obtidos diferentes tipos de calos de crescimento, de tal forma que plantas foram regeneradas a partir de calos de 13 espécies diferentes. Não haviam sido ainda relatadas culturas *in vitro* para 25 das espécies trabalhadas.
Palavras-chave: cultura de embrião, cultura de endosperma, espécies etnibotânicas, regeneração, aclimatização

INTRODUCTION

The genus *Passiflora* comprises several hundred species, mainly native of the South American tropics and rainforests, which are grouped into 21 subgenera. Some species (*P. edulis, P. quadrangularis, P. ligularis*) are widely studied for their economic importance and are chiefly cultivated for production of fruit juice. *P. incarnata* is reputed for its sedative properties and several other species are known for their ethnobotanical uses (see The Phytochemical and Ethnobotanical Databases, http://www.ars-grin.gov/duke/). However, the molecules responsible for these various activities are known only for a few species. Also, some *Passiflora* species have been described only recently (e.g. *P. trialata*, Feuillet & MacDougal, 1996).

For systematic study of the activities of secondary metabolites, a continuous source of material is necessary. For some *Passiflora* species, dehydrated seeds can be purchased, but for most, seeds have to be collected in the wild. Nonetheless, dehydrated seeds of many *Passiflora* species may require from many months up to two years to germinate (Vanderplank, *Passiflora* Society International Meeting, Rome, 15-16 September 2001). To alleviate these difficulties, an *in vitro* collection of *Passiflora* species, together with a greenhouse collection of regenerated plants was established.

Many *in vitro* culture techniques have been described for the *Passiflora* genus including regeneration from hypocotyl, leaves and cotyledons (Faria & Segura, 1997; Dornelas & Vieira, 1994), regeneration from leaf disks (Monteiro et al., 2000) and mesophyll and cotyledon-derived protoplasts (Dornelas & Vieira, 1993; Vaz d'Utra et al., 1993; Otoni et al., 1995, Anthony et al., 1999), regeneration after protoplast fusion (Dornelas et al., 1995), and micropropagation (Kawata et al., 1995). A mature endosperm culture has been reported for *P. foetida* (Mohamed et al., 1996). Embryo and endosperm culture from seeds of several *Passiflora* species mainly collected in the wild has been attempted in this study.

MATERIAL AND METHODS

Passiflora seeds, collected in the wild or produced in greenhouse, were kindly furnished by Dr. Maurizio Vecchia (Ripalta Cremasca, Cremona, Italy). For each species, four to 20 seeds were available. Mature seeds were surface sterilized with 70% ethanol for 10 min, followed by immersion in a sodium hypochlorite solution, containing 5% active chlorine for 70 min. During the first 10 min, samples were kept under vacuum. Seeds were extensively washed with sterile water, soaked overnight at 35°C, and then for 24 h at room temperature. They were then treated with sodium hypochlorite for 10 min and extensively washed prior to dissection. Embryos were extracted from seeds under a dissection microscope in a flow cabinet, and placed on media A and B; for some species, C or BG medium was also used (for media composition see Table 1). When solid parts of endosperm were present, they were explanted as well.

The following treatments depended on the response of the embryo: a) when embryo produced calli, these were maintained on B5+ or B5-2 medium (Table 1) depending on the species, and subcultured every 30 days; part of these calli were transferred to regenerating medium (REM) to regenerate shoots; rooting medium (RM) was used to obtain roots from shoots; b) when germination from explanted embryos occurred, part of the plantlets where cut into pieces and transferred to B5-2 medium to obtain undifferentiated calli, which were treated as above.

Part of the regenerated or germinated plantlets were transferred into Magenta vessels and grown *in vitro* in hormone-free MS medium (Murashige & Skoog, 1962) until both shoots and roots were well developed. The small plants were acclimatized in autoclave-sterilized soil for about three weeks at 100% humidity, and then transplanted into pots in a greenhouse.

RESULTS AND DISCUSSION

Embryo and endosperm culture: early events

Seeds of *Passiflora* genus vary greatly in size and shape. However, several common features are apparent, including hard seed coats surrounding a white, well-developed, straight embryo, with large flat cotyledons. A thin layer of endosperm, which can be ruminated, surrounds the embryo.

Endosperm and embryos extracted from seeds (Table 2) were grown in two different media, A and B (Table 1). The plant growth regulator and sucrose concentration of these media have been reported to induce undifferentiated callus formation (medium A) and to stimulate *in vitro* germination of zygotic embryos (medium B) in rice (Ko et al., 1983). Twenty six species responded to either A or B medium with embryo germination or callus formation (Table 2).

When a sufficient number of seeds were available, embryos of species that did not respond to A or B media were treated with medium containing gibberellic acid (BG medium, Table 2). Embryos of *P. mayarum*, *P. morifolia* and *P. subpeltata* germinated on this medium. *P. foetida* and *P. palmeri* were also tested in medium C, which induced embryo germination (*P. foetida*) and callus production from embryos (*P. palmeri*).

In most of the responsive species, the earlier modification observed in responding embryos was cotyledon greening, which occurred within one-three weeks of culture (data not shown), followed by cotyledon enlargement and opening (Figure 1a). This effect was independent of the composition of the growth medium.

The subsequent events depended on the final fate of the embryos: germinating embryos presented further cotyledon and root expansion (Figures 1a, b), occasionally accompanied by a small callus proliferation, especially at the hypocotyl-root border (Figure 1a), and cotyledon edges. Another group of embryos showed the on-

Table 1 - Media used for *Passiflora* culture

Medium	Salts, vitamins	Sucrose	2,4-D	Kinetin	NAA	GA	6BAP	IAA	IBA	Agar
		%	———————— μmol L^{-1} ————————							%
A	MS	3	13.6	9.3	-	-	-	-	-	0.9
B	MS	6	-	0.93	1.0	-	-	-	-	0.9
BG	MS	6	-	0.93	1.0	1.0	-	-	-	0.9
C	MS	2	2.25	-	-	-	1.1	1.4	-	0.8
REM	MS	2	2.25	-	-	-	1.1	-	2.46	0.8
RM	MS	2	-	-	-	-	-	-	2.46	0.8
B5+	B5	2	2.25	-	-	-	-	-	-	0.8
B5-2	B5	2	9.0	-	-	-	-	-	-	0.8

REM: regeneration medium; RM: rooting medium; MS: Murashige and Skoog's medium (Murashige & Skoog, 1962); B5: Gamborg's medium (Gamborg et al., 1968); 2.4-D: 2.4-dichlorophenoxyacetic acid; NAA: naphtalenacetic acid; GA: gibberellic acid; 6BAP: 6-benzylaminopurine; IAA: indoleacetic acid; IBA: indole-3-butyric acid.

Table 2 - Type of response of *Passiflora* species to different inducing media

Passiflora responsive species (subgenus)	Medium				Regeneration[a]
	A	B	C	BG	
**P.apetala (Decaloba)*	EG, CFE	EG	nt	nt	-
**P.auriculata (Decaloba)*	CFEM	CFEM	nt	nt	-
**P.candida (Astrophea)*	EG, CFEM	-	nt	nt	-
P.cincinnata (Passiflora)	CFE, CFEM	EG	nt	nt	+ (A,B)
**P.cirrhiflora (Polyanthea)*	CFEM	EG, CFEM	nt	nt	-
P.coccinea (Distephana)	CFEM	-	nt	nt	-
**P.coriacea (Decaloba)*	EG, CFEM	EG	nt	nt	+ (A)
**P.crenata (Passiflora)*	-	EG	nt	nt	-
P.foetida (Dysosmia)	-	EG	EG	nt	+ (C)
**P.garckei (Passiflora)*	-	CFEM	nt	nt	+ (B)
**P.glandulosa (Distephana)*	CFE	EG, CFEM	nt	nt	+ (B)
**P.mansii (Astrophea)*	CFE	EG	nt	nt	-
P.incarnata (Passiflora)	CFEM, CFE	CFEM	nt	nt	+ (A)
P.incarnata "alba" (Passiflora)	EG, CFEM	EG	nt	nt	+ (A)
**P.kawensis (Astrophea)*	EG	-	nt	nt	-
**P.mayarum (Passiflora)*	-	-	nt	EG	+(BG)
**P.morifolia (Decaloba)*	-	-	nt	EG, CFE	nt
**P.naviculata (Passiflora)*	-	EG	nt	nt	-
**P.nitida (Passiflora)*	CFE, CFEM	EG,CFE	nt	nt	-
**P.organensis (Decaloba)*	-	EG	nt	nt	-
**P.palmeri (Dysosmia)*	CFEM	-	EG, CFEM	nt	+ (C)
**P.platyloba (Passiflora)*	-	EG	nt	nt	nt
**P.rufa (Decaloba)*	CFEM	-	nt	nt	nt
**P.sicyoides (Decaloba)*	-	EG	nt	nt	-
**P.subpeltata (Passiflora)*	-	-	nt	EG	+(BG)
**P.subrotunda (Passiflora)*	EG, CFE	CFE	nt	nt	-
**P.tenuifila (Passiflora)*	CFEM	EG, CFEM	nt	nt	+ (B)
**P.trialata (Passiflora)*	CFEM	-	nt	nt	+ (A)
**P.tripartita (Tacsonia)*	EG, CFEM	EG	nt	nt	+ (A)
P.vespertilio (Decaloba)	-	EG	nt	EG	-

EG: embryo germination; CFE: callus formation from endosperm; CFEM callus formation from embryo; -: no response; nt: not tested; * tissue culture not previously reported. [a] +: plant regeneration was obtained under the conditions described in the results from material induced in the medium indicated in parentheses.

The following species did not respond to any of the tested media: *P.actinia (Passiflora), P.adenopoda (Decaloba), P.ambigua (Passiflora), P.amoena (Astrophea), P.aurantia (Decaloba), P.boenderi (Decaloba), P.cinnabarina (Decaloba), P.cuneata (Decaloba), P.edulis flavicarpa (Passiflora), P.elegans (Passiflora), P.fanchonae (Decaloba), P.gabrielliana (Passiflora), P.gibertii (Passiflora), P.guatemalensis (Decaloba), P.hahnii (Decaloba), P.lancetillensis (Deidamioides), P.laurifolia (Passiflora), P.ligularis (Passiflora), P.lutea (Decaloba), P. maliformis (Passiflora), P.manicata (Manicata), P.menispermifolia (Passiflora), P.mixta (Tacsonia), P.murucuja (Murucuja), P.pergrandis (Passiflora), P.pittieri (Astrophea), P.quadrangularis (Passiflora), P.serratifolia (Passiflora), P.serratodigitata (Passiflora), P.serrulata (Passiflora), P.suberosa (Decaloba).*

set of undifferentiated cell proliferation, especially from cotyledon edges and hypocotyl, which resulted in callus production.

Embryo germination occurred at a higher frequency on medium B (17 of 20 responsive species), while medium A ordinarily induced embryos to produce undifferentiated calli (17 of 18 responsive species), as expected (Ko et al., 1983). However, 7 of the 27 responsive species presented embryo germination in medium A, while 8 of the 27 responsive species presented calli on medium B. In particular, *P. kawensis* responded only to medium A with embryo germination.

In the case of accidental wounding during seed opening, isolated embryo organs were still able to grow,

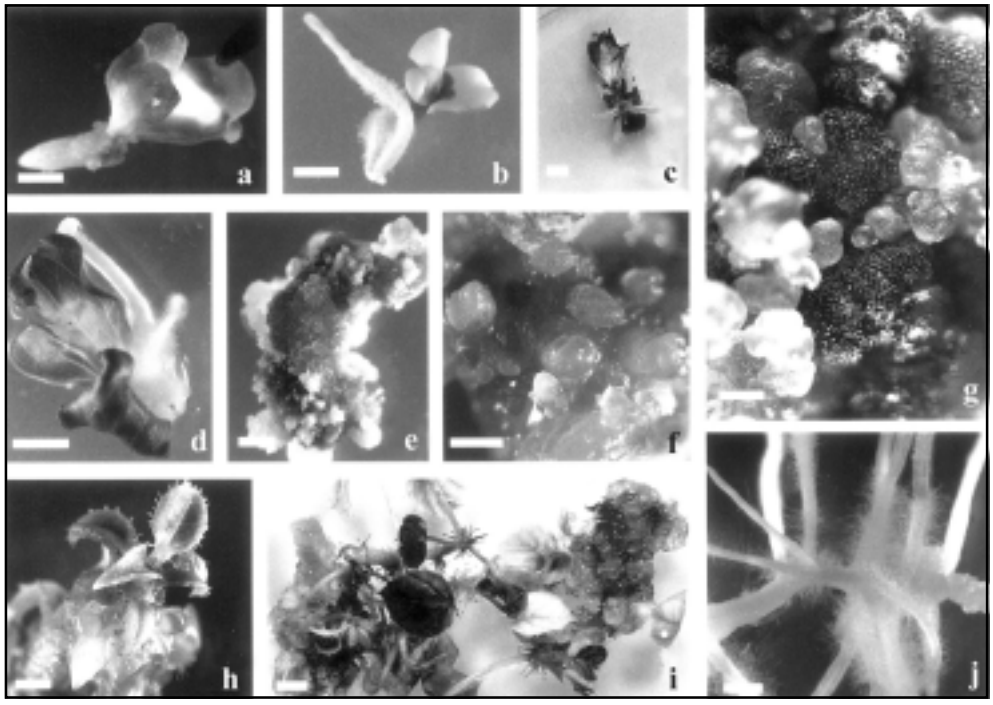

Figure 1 - *In vitro* culture of *Passiflora* embryos. Embryos *in vitro* germinated from a) *P. trialata* and b) *P. incarnata*, c) *P. foetida*: shoot germination in an embryo with partially coated root, d) *P. cincinnata*: isolated embryo shoot, with root induced on IBA containing medium, e) *P. garkei*: green, compact callus, f) *P. trialata*: green compact callus transferred to REM showing early regeneration response, g) *P. garkei*: green compact callus transferred to REM, showing the development of organized masses with epidermis-like purple layers. Well-developed shoots from regenerating calli of h) *P. palmeri* and i) *P. foetida*, j) *P. mayarum* branched roots emerging from white friable callus in callus maintaining B5+ medium. Bar a-e, h-j = 500 μm; f, g = 100 μm.

giving rise to shoots without roots and vice-versa (data not shown). Shoots grew independently from the root even in the case of incomplete excision of the embryo from seed coats, when residual seed coat pieces embedded the root (Figure 1c). It is reasonable to surmise that the failure of root germination depended on some dormancy factors within the seed coats that were still in tight contact with the root. The independent shoots were able to root on medium containing IBA (indole-3-butyric acid) (RM, see Table 1) (Figure 1d); the independent roots were cut and used to obtain calli in high 2.4-D (2.4-dichlorophenoxyacetic acid) medium (B5-2, Table 1).

Fragments of endosperm from mature seeds were also explanted in A, B, and BG media, resulting in undifferentiated white or yellow callus production for 7 of the 27 species in A, 2 of the 27 species in B, and 1 of the 4 species in BG (Table 2). Several species did not respond to any of the tested media (Table 2). However, for some unresponsive species only a very few seeds were available. The responsiveness of species to our culture conditions appeared to be independent on the taxonomic position, at least for the *Decaloba* and *Passiflora* subgenera, in which 8 of 19 and 12 of 27 species, respectively, showed some kind of response.

Callus culture and plant regeneration

Undifferentiated calli were spontaneously produced especially from embryos grown on medium A. Alternatively, pieces of embryo-derived hypocotyl or root were cut and transferred to high 2.4-D (B5-2 medium, Table 1) to obtain calli. Different embryos from a single species and even individual embryos produced calli with different characteristics (Table 3).

These calli were separated and grown on B5 medium containing 2.25 μmol L^{-1} 2.4-D and several homogeneous cell lines with different features were obtained (Table 3). Media with high 2.4-D (9 μmol L^{-1}) were chosen for species with very high morphogenetic capabilities, such as *P. foetida*, in order to maintain the undifferentiated callus.

Preliminary characterization has shown that calli have different morphogenetic potential, even within the same species. The growth rate of calli varied greatly among different species. The increase in weight of very low and fast growing species are detailed in Table 4. Green and compact calli were chosen as the best candidates to induce shoot regeneration (Figure 1e). For this purpose, calli were treated with medium containing 6-BAP (6-benzylaminopurine) (REM, Table 1). Regeneration was obtained after a transition period varying from

Table 3 - Characteristics of calli obtained from P*assiflora* responsive species

Specie	Characteristics of calli
P.apetala	c-d c-y f-y
P.auriculata	c-y c-g f-y
P.candida	c-d
P.cincinnata	c-d c-g cy
P.cirrhiflora	c-g c-y f-y f-w
P.coccinea	c-g cy f-g f-y f-w
P.coriacea	c-d c-g f-w f-d
P.crenata	c-g
P.foetida	c-d c-g c-y f-g f-y
P.garckei	c-g f-g f-y
P.glandulosa	c-w c-d
P.hahnii	c-d
P.incarnata	c-y c-g
P.incarnata "alba"	f-y f-g
P.kawensis	f-w
P.mayarum	c-g
P.morifolia	f-w c-g
P.nitida	c-g f-g f-y fw
P.palmeri	c-g c-d f-g f-y f-w
P.platyloba	f-w
P.rufa	c-g c-d c-y f-y f-w
P.sicyoides	c-d
P.subpeltata	c-w
P.subrotunda	c-d
P.tenuifila	c-d f-d f-y f-w
P.trialata	c-g c-d f-g f-w
P.tripartita	c-g c-w
P.vespertilio	c-w f-w

c: compact; f: friable; y: yellow; g: green; w: white d: dark yellow.

a few weeks to several months. During this period, green round masses that gradually developed an epidermis-like tissue appeared at the callus surface (Figures 1f, g). Successively, shoots emerged from these masses (Figure 1 h, i). Shoot regeneration was successful in 13 callus-forming species. Calli of *P. foetida* and *P. tenuifila* developed shoots spontaneously even without 6-BAP treatment.

Shoot regeneration was accompanied or followed by root regeneration in either MS hormone-free or IBA containing medium (RM, Table 1). In only one case (*P. palmeri*) the shoots did barely differentiate into roots, even in rooting medium and over a long period of time (several months).

In callus-maintaining medium, *P. mayarum* had spontaneous formation of long and branched roots from white friable calli (Figure. 1j). These roots were subsequently able to develop shoots in hormone-free medium. Calli obtained from endosperm have not yet been tested for their regeneration capabilities. However, regeneration from endosperm-derived callus has been described in *P. foetida* (Mohamed et al., 1996).

Table 4 - Weight (g) increase of selected P*assiflora* calli after 20 days of culture

Species (type of callus)	initial weight (t0)	final weight (t20)	t20/t0[a]
P. rufa (c-g)	0.578	1.980	3.43
P. apetala (f-w)	0.254	0.611	2.41
P. nitida (f-y)	0.260	3.962	15.24
P. garkei (f-y)	0.298	3.850	12.92
P. palmeri (f-y)	0.293	4.394	14.99

[a]ratio between final and initial weight.
P. rufa and *P. apetala* are representative of low-growing species, while *P. nitida*, *P. garkei* and *P. palmeri* are representative of fast growing species.

Plantlet acclimatization

Plantlets obtained from embryo germination or by regeneration were acclimatized and transplanted into pots and grown in the greenhouse, with a near 100% success rate, with the exception of *P. vespertilio*. In the greenhouse, mature plants of *P. foetida*, *P. tenuifila* and *P. coriacea* appeared to be fertile, spontaneously producing seed-containing fruits. *P. apetala* and *P. palmeri* produced normal flowers, although no fruits have yet been observed. Since plants were not hand-pollinated, this could simply be attributed to the absence of the proper pollinator in self-incompatible species. The other species have not reached sexual maturity yet.

In conclusion, embryo culture allowed the production of calli from 29 *Passiflora* species, and plant regeneration from 13 of these. For 25 of the responsive species, tissue culture had not been previously reported. Embryo culture represents a possible strategy to obtain *Passiflora* plants not only to provide a continuous source of material, but also for the conservation of endangered species.

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