Chapter 2

In Vitro Culture and Phytochemical Analysis of *Passiflora tenuifila* Killip and *Passiflora setacea* DC (Passifloraceae)

Jenny Sumara Sozo, Daniel Cuzziol Cruz, Ana Flavia Pavei, Isadora Medeiros da Costa Pereira, Marcia Wolfart, Fernanda Ramlov, Daiane Fiuza Montagner, Marcelo Maraschin, and Ana Maria Viana

Abstract

We have developed reproducible micropropagation, callus culture, phytochemical, and antioxidant analysis protocols for the wild passion fruit species *P. tenuifila*, and *P. setacea*, native to the Brazilian endangered biomes Atlantic Forest, Cerrado, and Caatinga, by using seeds and explants from seedlings and adult plants. Genotype and explant origin-linked differences are visible amongst the Passiflora species concerning callus production, total phenolics, and antioxidant activity. The protocols developed for screening phytochemicals and antioxidants in *P. tenuifila* and *P. setacea* callus extracts have shown their potential for phenolic production and antioxidant activity. The high level of phenolic compounds seems to account for the antioxidant activity of methanolic extracts of *P. tenuifila* derived from 45-day-old immature seed callus. The methanolic extracts of callus derived from *P. setacea* seedling leaf node and cotyledonary node explants have shown the highest antioxidant activity despite their lower content of phenolics, as compared to cotyledon callus extracts. The optimized micropropagation and callus culture protocols have great potential to use cell culture techniques for further vegetative propagation, in vitro germplasm conservation, and secondary metabolite production using biotic and abiotic elicitors.

Key words *Passiflora tenuifila*, *Passiflora setacea*, Micropropagation, Callus, Total phenolics, Antioxidant activity

1 Introduction

Passiflora setacea and Passiflora tenuifila are endemic to the endangered Brazilian biome Cerrado, Caatinga, and the Atlantic Forest. Their fruits have great potential for commercial use by nutritional, pharmaceutical, and cosmetic industries due to the high content of phenolic compounds which are antioxidants, especially *P. tenuifila* [1]. *P. tenuifila* is a potential source of resistant genes for genetic improvement as it is resistant to the pathogens *Xanthomonas axonopodis* pv. passiflorae and *Cladosporium herbarum*. The main constraints for plant production of several *Passiflora* species as *P. setacea*

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(occurring in Caatinga, Cerrado, Atlantic Forest) and *P. tenuifila* (occurring in Cerrado and Atlantic Forest) are the low seed germination rate, slow seedling emergence, and lack of efficient seed conservation protocols [2–4]. In vitro propagation techniques have been established for several *Passiflora* species such as *P. alata*, *P. caerulea*, *P. cincinnata*, *P. edulis*, *P. setacea*, *P. foetida*, and *P. suberosa*. These techniques have been used for the rapid multiplication of healthy and disease-free elite plants, source of fruits, and medicinal products as the antioxidants and other biologically active compounds [5–7]. However, plant tissue culture systems for secondary metabolite and antioxidant assessment have been established for *P. quadrangularis* [5] and *P. alata* Curtis [8, 9]. The protocols described herein are based on efficient and reproducible methods for *P. tenuifila* and *P. setacea* shoot culture initiation, callus culture establishment, and evaluation of callus phenolic content and antioxidant activity. We have optimized in vitro callus culture systems of *P. tenuifila* and *P. setacea* according to the antioxidant activity [10]. In vitro culture and antioxidant responses are genotype, explant type, and callus age dependent. The phytochemical and antioxidant screening carried out on *P. tenuifila* and *P. setacea* callus extracts demonstrates potent antioxidant activity.

2 Materials

2.1 Plant Material

1. Collect seeds from stock mother plants of *P. tenuifila* and *P. setacea* from the germplasm collection maintained at the experimental station, Embrapa Cerrados, Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Unidade Planaltina (Planaltina, DF, Brazil).

2.2 Culture Media

1. Murashige and Skoog [11] powder basal medium (MS), plant cell culture tested (Table 1).
2. 59 mM Sucrose grade I, plant cell culture tested.
3. 88.5 mM D-(+)-glucose, plant cell culture tested.
4. 88.5 mM D-(−)-fructose, plant cell culture tested.
5. 2 g/L Phytagel, plant cell culture tested.
6. Plant growth regulators for plant cell culture: gibberellic acid (GA_3), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D).

2.3 Extraction and Estimation of Total Phenolics

1. Folin-Ciocalteu reagent.
2. Gallic acid (*see* **Notes 1** and **2**).
3. 20 % (w/v) sodium carbonate solution: Prepare a stock solution and store at 4 °C.

Table 1
Murashige and Skoog basal medium composition

Component	Concentration (mL/L)
Ammonium nitrate	1650.0
Boric acid	6.2
Calcium chloride (anhydrous)[a]	332.2
Cobalt chloride·$6H_2O$	0.025
Cupric sulfate·$5H_2O$	0.025
Ethylenediaminetetracetic acid (disodium salt)·$2H_2O$	37.26
Ferrous sulfate·$7H_2O$	27.8
Magnesium sulfate[b]	180.7
Manganese sulfate	16.9
Molybdic acid (sodium salt)·$2H_2O$	0.25
Potassium iodide	0.83
Potassium nitrate	1900.0
Potassium phosphate monobasic	170.0
Zinc sulfate·$7H_2O$	8.6
Glycine (free base)	2.0
Myoinositol	100.0
Nicotinic acid (free acid)	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

[a]Original formula contains calcium chloride dihydrate at 440.0 mg/L
[b]Original formula contains magnesium sulfate heptahydrate at 370 mg/L

4. 80 % (v/v) methanol, high-performance liquid chromatography (HPLC) grade.
5. Ultraviolet-visible (UV-Vis) microplate reader.

2.4 Free Radical Scavenging Effect of 2,2-Diphenyl-1-picrylhydrazyl

1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) reagent (*see* **Note 3**).
2. 80 % (v/v/) methanol, high-performance liquid chromatography (HPLC) grade.
3. 3,5-Di-*tert*-4-butylhydroxytoluene analytical standard (BHT).
4. Ultraviolet-visible (UV-Vis) microplate reader.

3 Methods

3.1 Preparation of Culture Medium

1. Prepare standard aqueous solutions of plant growth regulators (*see* Subheading 2.2, **item 6**) by adding two drops of 1 M NaOH.
2. Prepare appropriate culture medium by adding the recommended concentration of the carbon source (*see* Tables 2, 3, 4, 5, 6, 7, and 8) together with plant growth regulators (*see* **Notes 4**, **5**, and **9**).
3. Adjust medium pH to 5.8 with 1 M NaOH before adding 2 g/L Phytagel. Dispense into 25 × 150 mm glass culture tubes (8 mL/tube) and autoclave at 121 °C for 18 min.

3.2 Culture Condition

1. Seal vessels with transparent polypropylene film (76 × 76 mm) and maintain the cultures at 25 °C, 70 % relative humidity (RH) under a 16-h photoperiod, photosynthetic photon flux of 20–25 μmol/m²/s supplied by fluorescent light tubes. In all protocols use these culture conditions.

Table 2
Composition of culture media used for shoot culture initiation and callus induction from different explant types of *Passiflora tenuifila*

In vitro process	Explant type	Explant origin	Culture medium	Carbon source	PGR	Induction (%)[a]	Callus texture
Shoot culture	Shoot tips	Ex vitro-germinated seedling	MS	59 mM sucrose	None	100	–
		Adult plant	MS	59 mM sucrose	None	100	–
Callus culture	Immature seeds	Developing fruits	MS	59 mM sucrose	2.5 μM 2,4-D	60	Friable
	Shoot tips	Adult plant	MS	59 mM sucrose	1.25–5 μM NAA	92	Friable
		Ex vitro-germinated seedling	MS	59 mM sucrose	1.25–5 μM NAA	100	Friable
	Stem segments	Shoot cultures from adult plant	MS	88.5 mM sucrose	2.5 μM NAA	100	Friable
		Shoot cultures from seedling	MS	88.5 mM sucrose	1.25 μM NAA or 2.5 μM 2,4-D	100	Friable
		Shoot cultures from seedling	MS	88.5 mM glucose	2.5 μM NAA	100	Friable
		Shoot cultures from seedling	MS	88.5 mM fructose	2.5 μM NAA	100	Friable

Note: Basic MS basal medium containing 2 mg/L Phytagel
[a]Values are means of 3 replicates of 15 explants each per treatment. The photosynthetic photon flux at culture level was 20–25 μmol/m²/s

Table 3
Composition of culture media used for in vitro shoot culture growth from seedling and adult plant shoot tips of *Passiflora tenuifila*

Explant origin	Culture medium	Carbon source (mM)	PGR type	PGR (μM)	Shoot induction (%)[x]	Rooting (%)[x]	No. of shoots [y]	Shoot length (cm)[y]	No. of leaf nodes[y]
Seedling	MS	59 mM sucrose	IBA	0	40	20 a	1 a	7.8 a	7 a
	MS	59 mM sucrose		1.25	100	50 b	1.3 a	11.8 b	19.5 b
	MS	59 mM sucrose		2.5	100	40 b	1.8 a	8.9 a	15.8 b
	MS	59 mM sucrose		5	100	40 b	1.2 a	7.5 a	15.2 b
Adult plant	MS	59 mM sucrose	GA_3	0	100	0	2.2 a	8.5 ab	7.2 a
	MS	59 mM sucrose		1.25	100	0	1.6 a	13.8 b	10.8 b
	MS	59 mM sucrose		2.5	100	0	2.2 a	5.3 a	11.6 b
	MS	59 mM sucrose		5	100	40 b	2 a	4.5 a	10.2 b

Note: Basic MS basal medium containing 2 mg/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

[x]Values are proportions of ten replicates per treatment

[y]Values ($n = 15$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 4
Composition of culture media used for in vitro biomass production of *P. tenuifila* callus derived from different explant types cultured in light

Explant origin	Culture medium	Carbon source type	Carbon source (mM)	PGR type	PGR (μM)	Callus FW (mg)[x]	Callus DW (mg)[x]
Immature seeds	MS	Sucrose	88.5	2,4-D	2.5	1383.8 ± 187.2 c	99.4 ± 7.5 c
Stem segments	MS	Sucrose	88.5	2,4-D	2.5	1021 ± 208.7 a	91.1 ± 11.19 b
Stem segments	MS	Sucrose	88.5	NAA	1.25	924.8 ± 185.3 a	80 ± 7.9 b b
Stem segments	MS	Glucose	88.5	NAA	2.5	1063.5 ± 109.7 b	55.4 ± 2.6 a
Stem segments	MS	Fructose	88.5	NAA	2.5	1082.4 ± 134.8 b	56.6 ± 3.8 a

Note: Basic MS basal medium containing 2 g/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

FW fresh weight, *DW* dry weight

[x]Values ($n = 10$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 5
Composition of culture media used for in vitro biomass production, total phenolics, and percentage of DPPH* inhibition of *P. tenuifila* callus extracts derived from different explant types cultured in light

Explant type	Culture medium	Carbon source type	Carbon source (mM)	PGR type	PGR (μM)	Culture period (days)	Total phenolics (μg GAE/g DW)[x]	DPPH* inhibition (%)[x]
Immature seeds	MS	Sucrose	88.5	2,4-D	2.5	45	20.41 ± 0.06 c	84 ± 0.60 c
Stem segments	MS	Sucrose	88.5	2,4-D	2.5	80	14.07 ± 0.03 a	21.72 ± 0.38 a
Stem segments	MS	Fructose	88.5	NAA	2.5	80	13.92 ± 0.03 a	31.83 ± 0.88 b
Stem segments	MS	Sucrose	88.5	2,4-D	2.5	80	17.28 ± 0.03 b	22.27 ± 0.25 a

Note: Basic MS basal medium containing 2 g/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m²/s
GAE gallic acid equivalent, *DW* dry weight
[x]Values ($n = 5$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 6
Composition of culture media used for shoot culture initiation and callus induction of *Passiflora setacea*

In vitro process	Explant type	Explant origin	Culture medium	Sucrose (mM)	2,4-D (μM)	Induction (%)[x]	Callus texture
Shoot culture initiation	Shoot tips	Seedling (ex vitro)	MS	59	0	100	–
Callus culture initiation	Root	Seedling (in vitro)	MS	59	5	100	Friable
	Hypocotyl	Seedling (in vitro)	MS	59	5	100	Friable
	Cotyledonary node	Seedling (in vitro)	MS	59	2.5	100	Compact
	Leaf node	Seedling (in vitro)	MS	59	2.5	100	Friable
	Cotyledon	Seedling (in vitro)	MS	59	5	100	Compact

Note: Basic MS basal medium containing 2 mg/L Phytagel
[x]Values are means of three replicates of ten explants each per treatment. The photosynthetic photon flux at culture level was 20–25 μmol/m²/s

Table 7
Composition of culture media used for in vitro shoot growth from shoot tip culture of *P. setacea*

Culture medium	Carbon source type	Carbon source (mM)	IBA (μM)	Shoot induction (%)[x]	Rooting (%)[x]	No. of shoots[y]	Shoot length (cm)[y]	No. of leaf nodes[y]
MS	Sucrose	59	0	100	0	1.4 ab	5.4 b	12.6 b
MS	Sucrose	59	1.25	100	60	1.6 b	8.5 c	17.8 c
MS	Sucrose	59	2.5	100	0	1 a	6.7 b	10 b
MS	sucrose	59	5	100	0	1.2 a	4.2 a	5.8 a

Note: Basic MS basal medium containing 2 mg/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

[x]Values are proportions of ten replicates per treatment

[y]Values ($n=15$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 8
Composition of culture media used for in vitro biomass production, total phenolics, and percentage of DPPH* inhibition of *P. setacea* callus extracts derived from different seedling explant types cultured in light

Explant type	Culture medium	Sucrose (mM)	2,4-D (μM)	Culture period (days)	Callus FW (mg)[x]	Callus DW (mg)[x]	Total phenolics (μg GAE/g DW)[y]	DPPH* inhibition (%)[y]
Root	MS	88.5	2.5	45	1822.0 ± 271.0 c	82.8 ± 6.7 c	8.47 ± 0.02 a	55.51 ± 2.00 a
Cotyledonary node	MS	88.5	2.5	45	1347.0 ± 204.0 b	64.4 ± 8.1 a	12.34 ± 0.02 b	77.66 ± 0.90 c
Hypocotyl	MS	88.5	2.5	45	1044.8 ± 163.0 ab	61.4 ± 10.4 a	10.11 ± 0.01 b	63.84 ± 1.89 b
Leaf node	MS	88.5	2.5	45	1107.6 ± 200.8 b	61.4 ± 10.4 a	11.10 ± 0.03 b	88.17 ± 0.50 d
Cotyledon	MS	88.5	2.5	45	960.0 ± 207.3 a	70.2 ± 10.4 b	17.26 ± 0.04 c	52.50 ± 1.17 a

Note: Basic MS basal medium containing 2 g/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

GAE gallic acid equivalent, *FW* fresh weight, *DW* dry weight

[x]Values ($n=10$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

[y]Values ($n=5$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

3.3 Establishment of Shoot Cultures of P. tenuifila

3.3.1 Ex Vitro Seed Germination

1. Collect the seeds from fresh mature fruits of *P. tenuifila* immediately after harvesting.
2. Remove the seed arils.
3. Immerse *P. tenuifila* seeds completely in 2 mL 2.5 % (w/v) gibberellic acid aqueous solution at 25 °C for 5 days (*see* **Note 5**).
4. Remove *P. tenuifila* seeds from the GA_3 aqueous solution and sow in 50 mL plastic cups containing 28 g soil. Add 8 mL distilled water.
5. Place plastic cups in 23.5 cm × 16.9 cm × 10 cm polystyrene trays.
6. Maintain the trays either at 25 °C or in the greenhouse with a natural photoperiod at 16 °C during the night and 40 °C during the day (*see* **Note 5**).

3.3.2 Surface Sterilization of Seedling and Adult Plant Shoot Tips

1. Excise shoot tips, 3–4 cm in length comprising one apical bud and two axillary buds, from 45- to 60-day-old seedlings (*see* Fig. 1a) or greenhouse-grown 15-month-old adult *P. tenuifila* plants.
2. Rinse shoot tips in 100 mL tap water, containing 2–3 drops of commercial detergent.
3. Wash four times with distilled water.
4. Surface sterilize shoot tips of *P. tenuifila* seedlings for 1 min 30 s in 70 % (v/v) alcohol, rinse for 3 min in sterile distilled water, and immerse in commercial bleach (2.5 % active chlorine) with 2–3 drops of Tween 20 for 2 min 30 s.
5. Surface sterilize shoot tips of *P. tenuifila* adult plants for 3 min in 70 % (v/v) alcohol, rinse for 3 min in sterile distilled water, and immerse in commercial bleach (2.5 % active chlorine) with 2–3 drops of Tween 20 for 4 min.
6. Rinse the shoot tips three times each for 10 min in sterile distilled water.

3.3.3 Shoot Initiation and Multiplication

1. Place surface-sterilized shoot tips of *P. tenuifila* vertically on the MS basal medium containing 59 mM sucrose and 2 g/L Phytagel (*see* **Note 5**, Fig. 1b).
2. Promote in vitro shoot elongation by transferring the 30-day-old in vitro shoots to MS basal medium with 59 mM sucrose, 2 g/L Phytagel, and either 1.25 μM IBA (*P. tenuifila* seedling shoot tips) or 1.25 μM GA_3 (*P. tenuifila* adult plant shoot tips) (*see* **Note 7**) (Fig. 1c).
3. Evaluate the percentage of shoot formation, number of shoots, shoot length, and number of nodes initiated per explant after 12 weeks. The results for shoot induction and growth of *P. tenuifila* are shown in Tables 2 and 3.

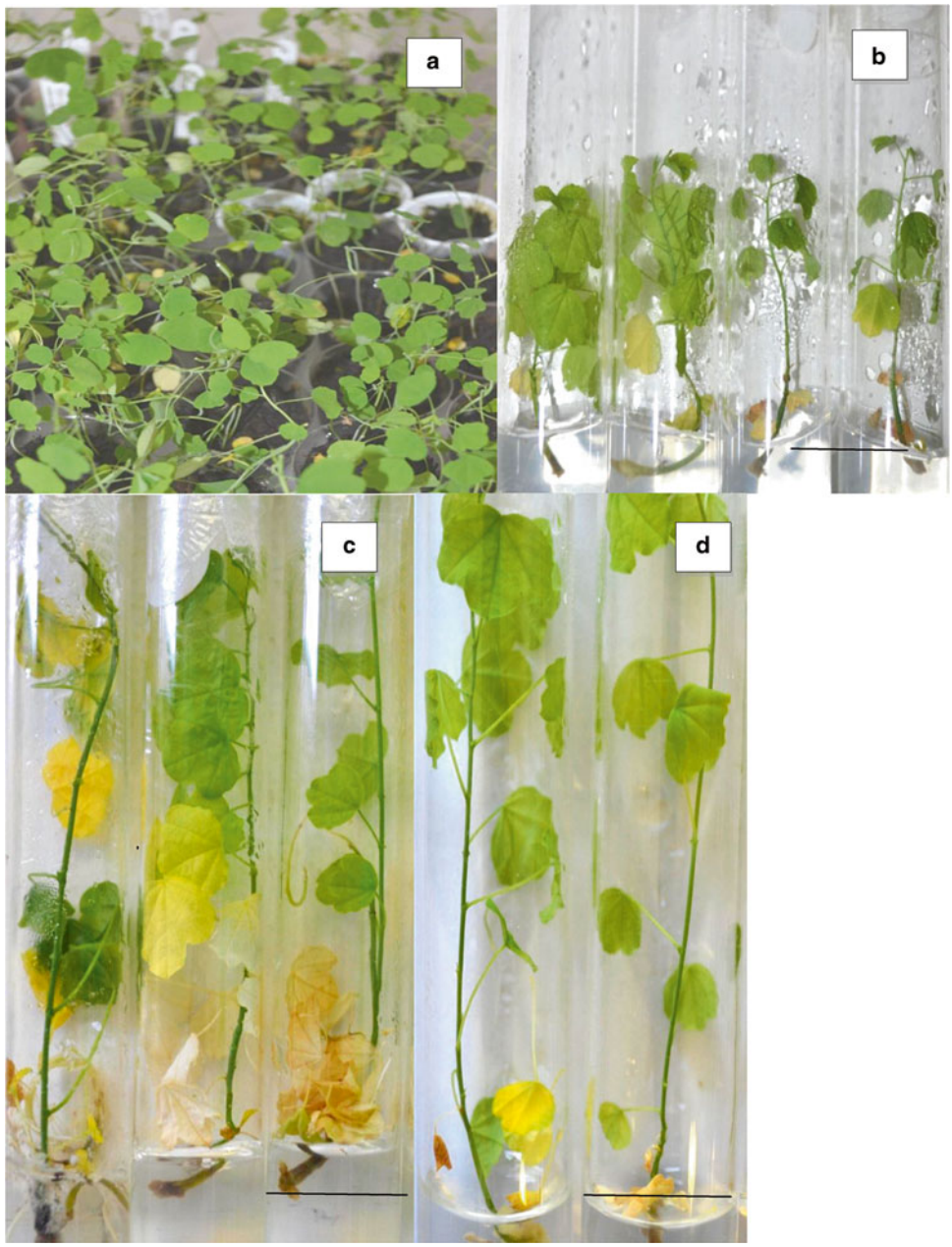

Fig. 1 Ex vitro-germinated seedlings of *Passiflora tenuifila* (**a**). In vitro shoot cultures originated from seedling shoot tip explants of *Passiflora tenuifila* cultured on MS basal medium with 2 mg/L Phytagel, 59 mM sucrose (**b**); 59 mM sucrose and 1.25 μM GA_3 (**c**) after 90 days; 88.5 mM glucose and 1.25 μM IBA (**d**) after 45 days. Bars = 25 mm

4. For shoot multiplication and shoot culture stock maintenance remove 2 cm long shoot tips and single-leaf node explants (ca. 1.0 cm long) from the 8-week-old *P. tenuifila* plantlets originated from the shoot tips cultured according to the procedures 1–2 (*see* **Note 8**).

5. Place explants vertically on the MS basal medium supplemented with 88.5 mM glucose, 2 g/L Phytagel, and 1.25 μM IBA. The results for in vitro shoot growth of *P. tenuifila* are shown in Fig. 1d (*see* **Note 8**).
6. Subculture after every 60-day culture cycle.

3.4 Establishment of Callus Cultures

3.4.1 Establishment of Callus Cultures from Immature Seeds

1. Harvest immature fruits (4-week-old after anthesis) from *P. tenuifila* greenhouse-grown plants.
2. Rinse fruits in 100 mL tap water containing 2–3 drops of commercial detergent.
3. Surface sterilize fruits for 5 min in commercial alcohol in the flow cabinet.
4. Remove the seeds and place them on MS basal medium amended with 59 mM sucrose, 2 g/L Phytagel, and 2.5 μM 2,4-D (*see* **Notes 9** and **10**).
5. After 45 days evaluate the callus induction. Example results of callus induction are shown in Table 2, Fig. 2a.
6. Subculture callus (ca. 50 mg fresh weight) every 30-day cycle to MS basal medium containing 88.5 mM sucrose, 2 g/L Phytagel, and 2.5 μM 2,4-D (*see* **Note 11**).
7. After 45 days evaluate callus fresh and dry mass. The results for callus growth are shown in Table 4, Fig. 2b (*see* **Notes 10** and **11**).

3.4.2 Establishment of Callus Cultures from Seedling and Adult Plant Shoot Tips

1. Surface sterilize shoot tips of *P. tenuifila* following the procedures described in Subheading 3.3.2.
2. Place explants vertically on the MS basal medium supplemented with 59 mM sucrose, 2 g/L Phytagel, and 1.25 μM NAA (*see* **Note 9**).
3. Evaluate the percentage of callus induction and fresh and dry mass after 45 days. The results for callus induction are shown in Table 2, Fig. 2c–e (*see* **Notes 10** and **11**).

3.4.3 Establishment of Callus Cultures from Stem Segments of In Vitro-Formed Shoots

1. Remove stem segments (2–3 mm in length) from 60-day-old shoot cultures of *P. tenuifila* established according to the procedures described in Subheading 3.3.2 (Fig. 1).
2. Place the explants on MS basal medium containing 88.5 mM of either sucrose, glucose, or fructose, 2 g/L Phytagel, and either 1.25–2.5 μM NAA or 2,4-D (*see* **Note 9**).
3. After 45 days, evaluate the percentage of callus induction, fresh mass, and dry mass. The results for callus induction and growth are shown in Tables 2 and 4, Fig. 2f (*see* **Notes 10** and **11**).

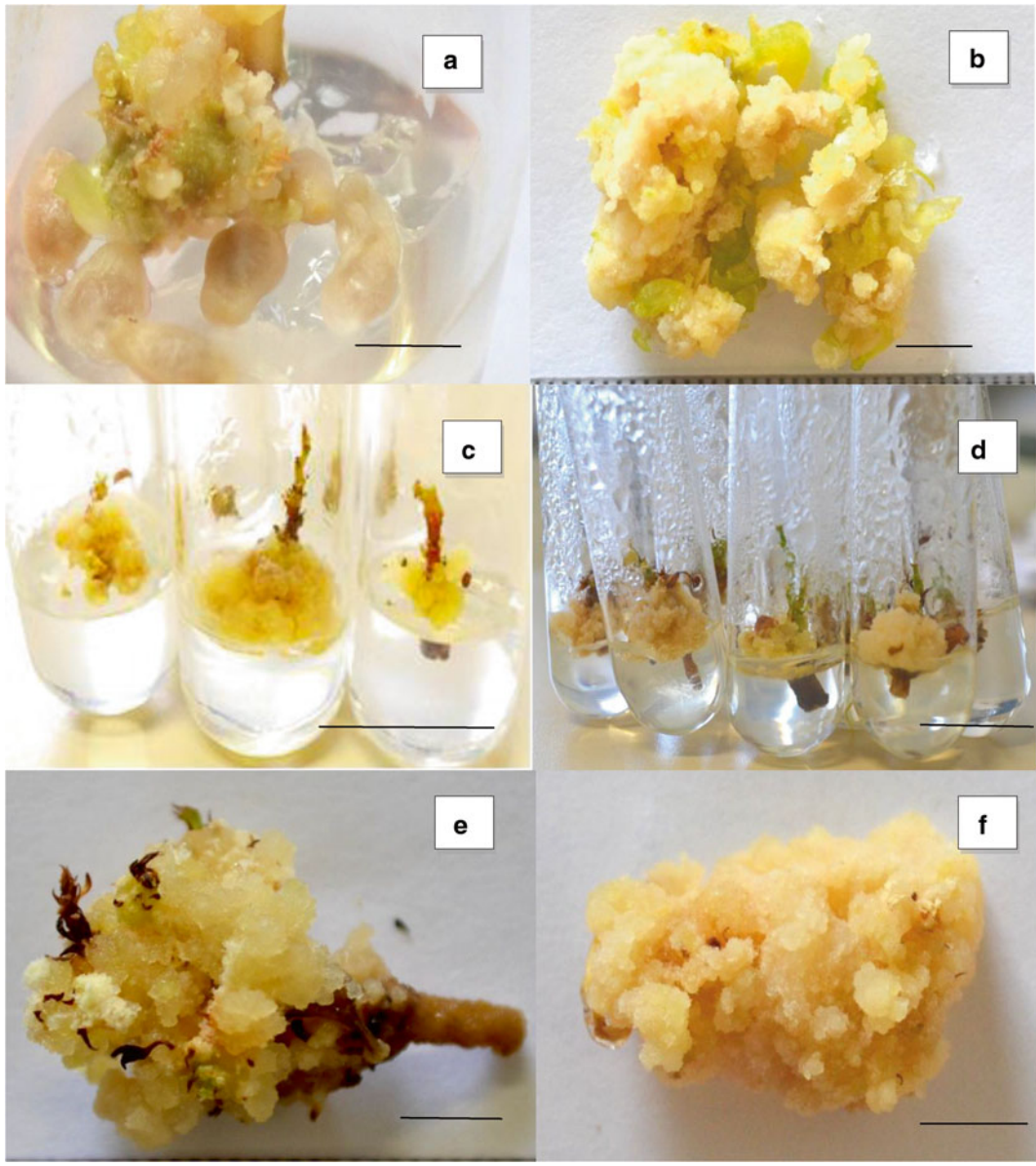

Fig. 2 Callus originated from immature seeds (**a**, **b**), from shoot tips of seedlings (**c**), adult plants (**d**, **e**), and stem segment explants of in vitro-formed shoots (**f**) of *Passiflora tenuifila* cultured on MS basal medium with 59 mM sucrose, 2 mg/L Phytagel, and 2.5 μM 2,4-D (**a**, **b**); 2.5 μM NAA (**c**–**e**); or 88.5 mM sucrose and 2.5 μM 2,4-D (**f**), after 50-day culture. Bars = 5 mm (**a**, **b**, **e**, **f**). Bars = 25 mm (**c**, **d**)

3.5 Establishment of Shoot Cultures of P. setacea

3.5.1 Ex Vitro Seed Germination

1. Collect the seeds from fresh mature fruits of *P. setacea* immediately after harvesting.
2. Remove the seed arils.
3. Sow *P. setacea* seeds in 50 mL plastic cups containing 28 g soil. Add 8 mL distilled water.
4. Place plastic cups in 23.5 cm × 16.9 cm × 10 cm polystyrene trays.

5. Keep the trays in the greenhouse with natural photoperiod and temperatures of 16 °C during the night and 40 °C during the day (*see* **Note 6**).

3.5.2 Shoot Initiation and Multiplication

1. Surface sterilize ex vitro-grown seedling shoot tips of *P. setacea* (*see* Subheading 3.3.2, **steps 1–4**; Fig. 3a).
2. Place surface-sterilized shoot tips of *P. setacea* vertically on the MS basal medium supplemented with 59 mM sucrose and 2 g/L Phytagel (*see* **Note 5**).
3. Evaluate the percentage of shoot formation, number of shoots, shoot length, and number of nodes initiated per explant after 12 weeks. The results of shoot induction and growth are shown in Tables 6 and 7, Fig. 3b (*see* **Note 12**).
4. For shoot elongation, multiplication and maintenance of shoot culture stocks of *P. setacea* follow the procedures described in Subheading 3.3.3, **steps 4–6** (*see* **Note 8**). The results for in vitro shoot growth of *P. setacea* are shown in Fig. 3c (*see* **Note 8**).

3.6 Establishment of Callus Cultures

3.6.1 Seed Surface Sterilization and In Vitro Seed Germination of *P. setacea*

1. Rinse seeds of selected *P. setacea* plants in 100 mL tap water with 2–3 drops of commercial detergent, and wash four times with distilled water.
2. Surface sterilize seeds for 10 min in commercial bleach (2.5 % active chlorine). Add 2–3 drops of Tween 20.
3. Rinse three times for 10 min in sterile distilled water and culture the seeds on MS basal medium supplemented with 59 mM sucrose and 2 g/L Phytagel (*see* **Note 6**).

3.6.2 Establishment of Callus Cultures from Axenic Seedling Explants

1. Remove segments (1 cm long) of either root, hypocotyl, epicotyl, cotyledonary node, or leaf node obtained from 60-day-old aseptically grown seedlings of *P. setacea* (Fig. 3d).
2. Place the explants horizontally on MS basal medium with 88.5 mM sucrose, 2 g/L Phytagel, and 2.5 μM 2,4-D (*see* **Note 13**).
3. After 45 days, take callus fresh and dry weight. The results of callus induction are shown in Table 6 (*see* **Notes 11** and **12**).
4. Subculture callus at 30-day interval by following the procedures described in Subheading 3.4.1, **step 6**. The results for callus growth are shown in Table 8, Fig. 4 (*see* **Notes 10** and **11**).

3.7 Phytochemical Analysis and Antioxidant DPPH Test

3.7.1 Preparation of Callus Extract for Estimation of Total Phenolic Compounds

1. Grind 1 g fresh callus material in 10 mL 80 % (v/v) methanol in water and leave the extract for 1 h in the darkness.
2. Centrifuge contents at 11.76×*g* for 5 min. The supernatant solution is filtered under vacuum into a volumetric flask and the filtrate is saved.

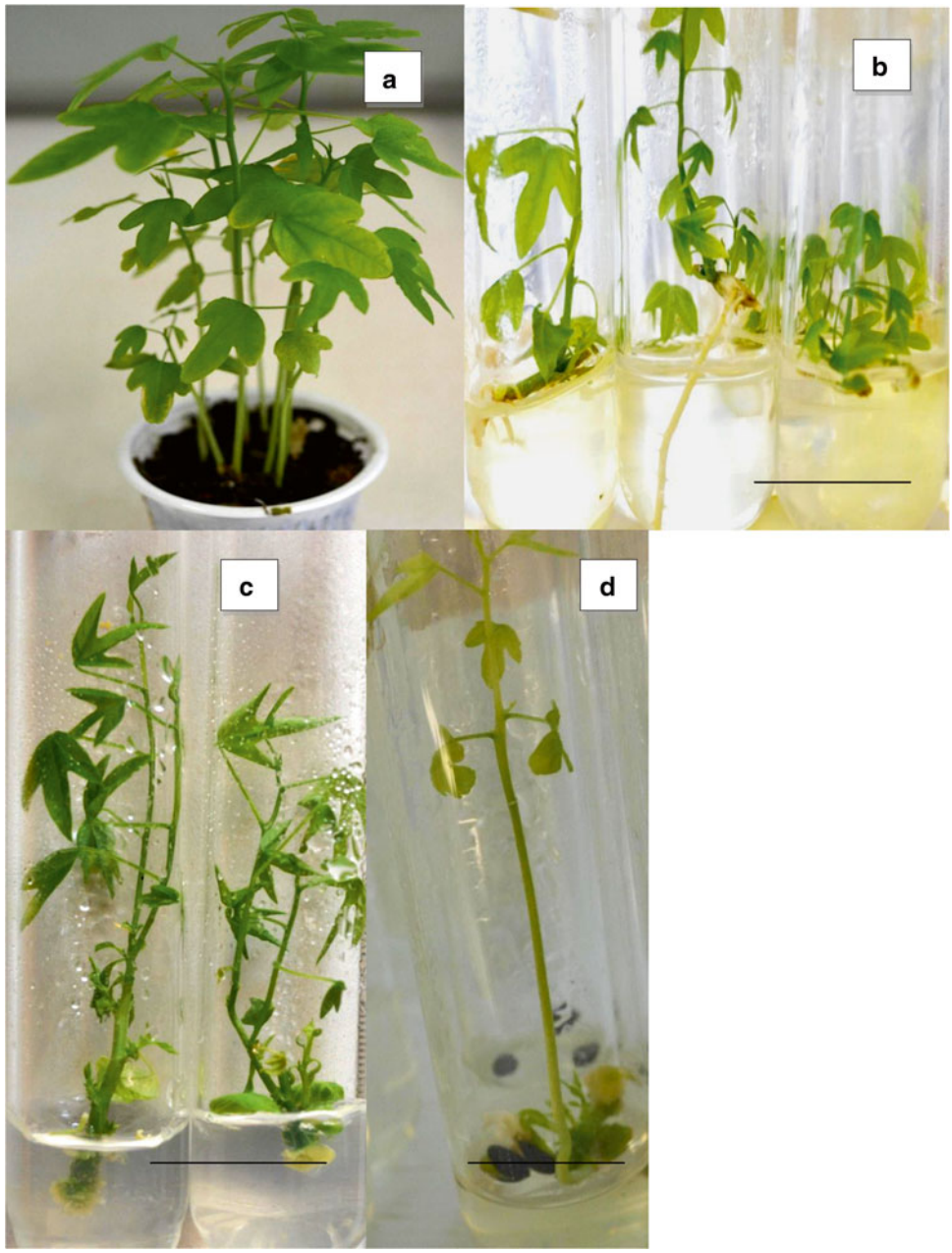

Fig. 3 Ex vitro-germinated seedlings of *Passiflora setacea* (**a**). In vitro shoots of *Passiflora setacea* originated from seedling shoot tip explants cultured on MS basal medium with 2 mg/L Phytagel and either 59 mM sucrose (**b**, **c**) or 88.5 mM glucose μM IBA (**d**) after 45 days. In vitro-germinated seedling of *Passiflora setacea*. Bars = 25 mm

3.7.2 Estimation of Total Phenolics Contents in the Callus Extract

1. Mix 40 μL methanolic extract with 3.16 mL deionized water and add 200 μL 10 % Folin-Ciocalteu reagent.

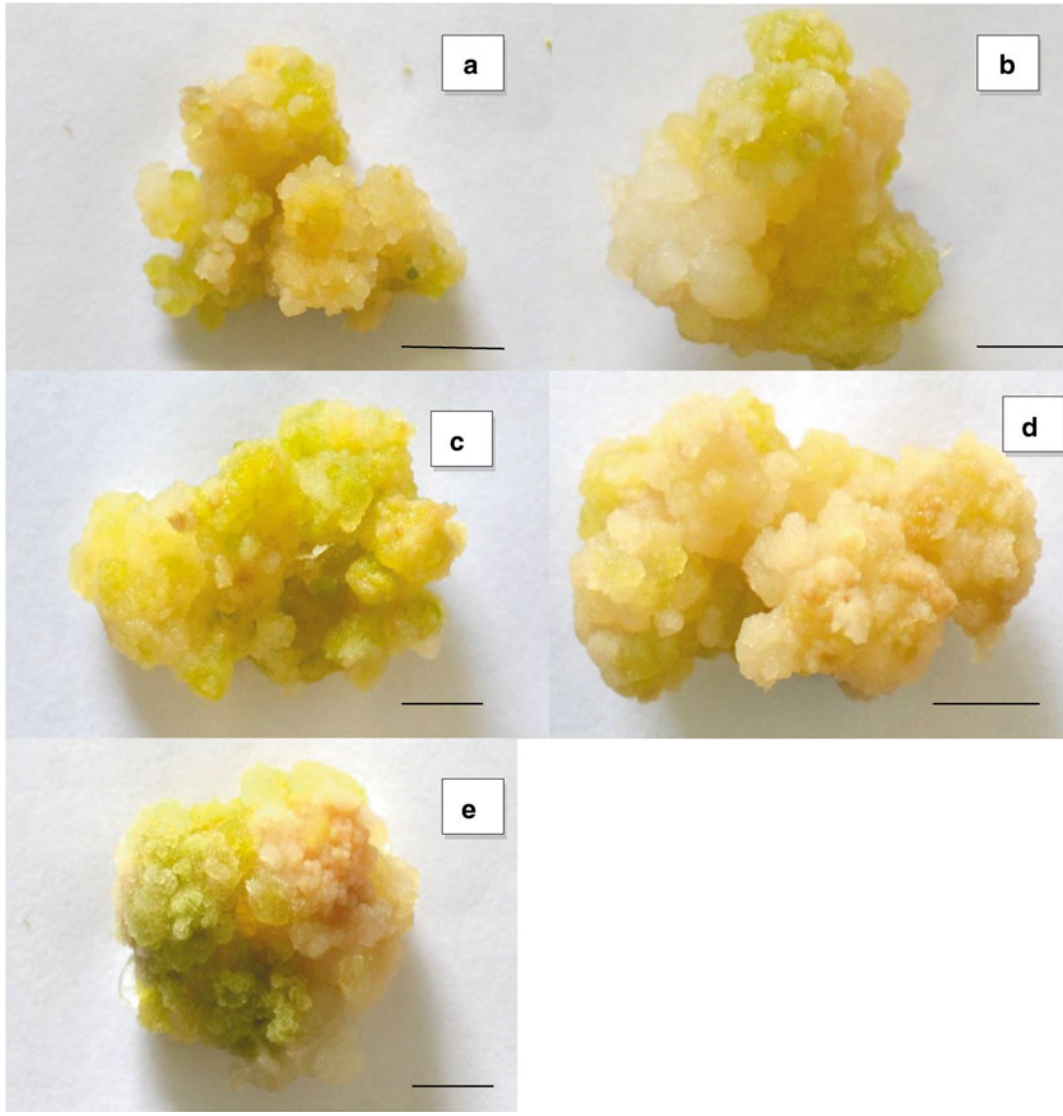

Fig. 4 Callus originated from roots (**a**), hypocotyl (**b**), cotyledonary node (**c**), leaf node (**d**), cotyledon (**e**) of in vitro-cultured seedlings of *Passiflora setacea* cultured on MS basal medium with 88.5 mM sucrose, 2 mg/L Phytagel, and 2.5 μM 2,4-D, after 45-day culture. Bars = 5 mm

2. After 6 min the reaction is neutralized with 600 μL 20 % sodium carbonate solution. The color will develop after incubation for 2 h at the room temperature in the darkness.
3. 300 μL of each sample and control are transferred to 96-well microplate and the absorbance is detected at 760 nm on a UV-visible microplate reader. The measurements are compared to the standard curve for gallic acid (0–1000 μg/mL) (*see* **Notes 1** and **2**).

4. The results for total phenolics are expressed as microgram of gallic acid equivalent per gram of dry callus. The results of total phenolic contents in callus of *P. tenuifila* (Table 5) and *P. setacea* (Table 8) are shown (*see* **Note 14**).

3.7.3 Free Radical Scavenging Effect on 2,2-Diphenyl-1-picrylhydrazyl

1. Mix samples of 10 μL methanolic extract with 290 μL 0.05 mM methanolic solution of DPPH in a 96-well microplate (*see* **Note 3**).
2. A DPPH blank sample, without extract, containing 10 μL 80 % methanol and 290 μL DPPH solutions is prepared and assayed as control. The positive control is the DPPH solution plus *tert*-butylhydroxytoluene (BHT).
3. After incubation in the darkness at room temperature for 2 h, the absorbance of the reaction mixture is measured at 515 nm using a UV-visible microplate reader.
4. The percentage decrease in the absorbance at 515 nm is recorded for each sample and the percentage of quenching of the DPPH* radical is calculated on the basis of the observed decrease of the radical according to the formula DPPH* inhibition percentage $= [(A_{\mathrm{DPPH}} - A_{\mathrm{Extr}})/A_{\mathrm{DPPH}}] \times 100$ where A_{DPPH} is the absorbance value of the DPPH blank sample (control) and A_{Extr} is the absorbance value in the presence of the extract. The results for the percentage of DPPH scavenged of callus extracts of *P. tenuifila* (Table 5) and *P. setacea* (Table 8) are shown (*see* **Note 15**).

4 Notes

1. Prepare a stock solution of gallic acid by dissolving 100 mg gallic acid in 100 mL deionized distilled water. Firstly dissolve the reagent in 1 mL 80 % methanol and increase the volume to 100 mL by adding deionized distilled water. Store this stock solution in an amber glass flask in a refrigerator to use as fresh working standards. Stock solution should be maintained at room temperature before use. Analyze total phenolics in the callus extract spectrophotometrically by using Folin-Ciocalteu reagent [12].
2. Prepare working standards of 50–1000 μg/mL standard gallic acid solution. Total phenolics is expressed as microgram of gallic acid equivalent per gram of dry extract (μg GAE/g) using a standard curve (0–1000 μg/mL) of gallic acid.
3. Prepare fresh 0.05 mM DPPH* stock solution in 80 % methanol (w/v) and store in the darkness at 4 °C in a flask, covered with aluminum foil. DPPH radical has been widely used to evaluate the free radical scavenging capacity (antioxidant activity) of plant and microbial extracts [13, 14].

4. The media and plant growth regulator solutions are prepared in double-distilled water. Adjustment of pH is done before the addition of Phytagel. Store all the stock solutions at 4 °C until use.
5. To optimize the *Passiflora tenuifila* and *P. setacea* shoot and callus culture protocols, determine the most appropriate concentration of sucrose, glucose, IBA, GA_3, NAA, and 2,4-D. The optimum culture conditions for shoot culture initiation and growth are shown in tables (*see* Tables 2, 3, 6, and 7).
6. *Passiflora tenuifila* seeds fail to germinate in vitro. Seeds can be germinated in soil either at 25 °C or in the greenhouse (natural day light, minimum temperature 16 °C, maximum temperature 40 °C), after immersion for 5 days in 2.5 % GA_3 aqueous solution. The germination rates of the GA_3-treated seeds are 80–90 % within 20 days and mechanical scarification was unnecessary. We have successfully modified the originally proposed *P. alata* seed germination method [7].
7. *P. tenuifila* in vitro shoots are produced in 90–100 % shoot tips originated either from seedling or adult plant. IBA (for seedling shoot tip) and GA_3 (for adult plant shoot tip) are effective promoters of in vitro shoot elongation and growth (*see* Table 3). The multiplication rates varied from 10 to 20 propagules per culture cycle depending on the shoot tip origin. Rooting is achieved in 40–50 % in vitro shoots cultured in IBA-containing medium. After 8 weeks of culture, in vitro shoots formed highest rooting rate (*see* Table 3).
8. *P. tenuifila* and *P. setacea* shoot culture stocks are successfully maintained by subculturing shoot tips and leaf node segments on MS basal medium containing 88.5 mM glucose, 1.25 μM IBA, and 2 g/L Phytagel for 24 months without decreasing the shoot proliferation capacity (*see* Figs. 1 and 3) (8-week subculture time).
9. The best culture conditions for *P. tenuifila* callus induction (Table 2) and growth (Table 4) are shown. NAA is effective for callus induction from shoot tips while 2,4-D is effective for callus induction from immature seeds.
10. Consistent biomass (fresh and dry mass) is produced by the callus culture systems developed for *P. tenuifila* and maximum callus dry mass is obtained from immature seed-derived callus after ca. 45-day subculture cycle (*see* Table 5). *P. setacea* callus growth is affected by explant type and consistent callus biomass (fresh and dry mass) is produced by all callus culture systems developed for *P. setacea*. Callus derived from root and cotyledon segments explants shows the highest biomass accumulation (*see* Table 8). Maximum callus biomass is obtained after a 30–45-day subculture cycle.
11. The subculture of *P. tenuifila* callus cultures originated from in vitro-formed shoot stem segments and from shoot tips, either

from seedling or adult plants, is not as effective as the subculture of immature seed-derived callus. The maintenance of *P. tenuifila* immature seed callus and *P. setacea* seedling explant callus culture stocks is carried out successfully for at least 18 months through periodic subculture (30-day time) on MS basal medium supplemented with 88.5 mM sucrose, 2.5 μM 2,4-D, and 2 mg/L Phytagel (*see* Tables 4 and 8).

12. *P. setacea* in vitro shoots are produced in 100 % explants, originated from seedling shoot tips. Addition of 1.25 μM IBA efficiently promotes in vitro shoot growth (number of shoots, shoot length, and number of nodes/micro shot) and rooting (*see* Table 7). The multiplication rate is ca. 15–18 propagules per culture cycle. Rooting is achieved in 60 % of the in vitro shoots. Approximately 8-week time is required to achieve the highest rooting rate from *P. setacea* in vitro shoots (*see* Table 7).
13. The best culture conditions for *P. setacea* callus induction (Table 6) and growth (Table 8) are shown. Maximum callus induction rate (100 %) was achieved from all explant types.
14. The levels of total phenolic compounds in *P. tenuifila* callus extracts are shown in Table 5. Callus age and explant-type linked differences are observed. The analysis shows that phenolic compound contents are higher in 45-day-old callus originated from immature seeds of *P. tenuifila*. The levels of total phenolic compounds in *P. setacea* callus extracts are shown in Table 8. The phytochemical analysis of callus extracts reveals explant-type linked differences and suggests that phenolic compound contents are superior in callus originated from cotyledon segments.
15. The antioxidant activity of *P. tenuifila* callus extract is explant type and callus age dependent. The highest antioxidant activity is observed in callus originated from immature seeds which show the highest levels of total phenolics (*see* Table 5). The DPPH* test of *P. setacea* callus extracts also reveals explant-type linked differences and the highest antioxidant activity is detected in leaf node callus. Therefore, the maximum level of antioxidant activity is not dependent on the highest content of phenolic compounds (*see* Table 8).

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