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Review Article

Progress in micropropagation of *Passiflora* spp. to produce medicinal plants: a mini-review

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ABSTRACT

Micropropagation of *Passiflora* species and its hybrids may play an important role in the production of healthy and disease-free plants which can be a source of medicinal herbal products, nutritional fruits and ornamental flowers. The rapid multiplication of elite plants to obtain pharmacognostic material, containing valuable flavonoid C-glycosides, is possible by usingcontrolled *in vitro* conditions, constituents of the medium and the interactions of plant growth regulators (1-naphtaleneacetic acid, benzyladenine, gibberellin GA_3,kinetin, indole-3-acetyl-L-aspartic acid, indole-3-butyric acid, thidiazuron) and influencing various chemical additives (silver nitrate, coconut water, activated charcoal). Investigations of specific requirements during stages of micropropagation, such as the establishment of primary cultures (including type of explants, age of donor plant), shoot multiplication (by direct and indirect organogenesis and embryogenesis), rooting and acclimatization of regenerated plants are summarized in this review. The following species were recently studied for micropropagation: *P. alata, P. caerulea, P. cincinnata, P. edulis, P. foetida, P. setacea, P. suberosa*. It seems that for awide range of applications of *in vitro* clones of *Passiflora*, interdisciplinary studies including genetic and phytochemical aspects are needed.

Introduction

Passiflora species have a long history of use since the discovery of these plants by Spanish missionaries in South America. These plants belong to the family Passifloraceae, which contains manyexoticvines (sixteen genera and 650 species) providing valuableraw materials to the present day worldwide (Wiart, 2006). Species of *Passiflora* and its hybrids have beautiful ornamental flowers and some of them are cultivated. Currently, the high demand for the edible fruits is observed for more than sixty *Passiflora* species. *Passiflora* plants are used in traditional medicine not only in South America, but also in the Netherlands, Spain and Italy (Patel et al., 2009; 2011). The leaf extracts of *P. incarnata, P. edulis* and *P. alata* have been most extensively investigated (Zucolotto et al., 2011), but various *Passiflora* species are very attractive both for the horticultural sector as well as for the herbal and pharmaceutical industry due to their beautiful flowers, edible fruits and the presence of valuable bioactive compounds. These flavonoid glycosidesare present in high amounts in most of the various species *Passiflora*, which differ in the content and concentration of derivatives of apigenin (*i.e.* apigenin-6-C-rhamnosyl-8-C-arabinoside,apigenin-7-O-diglucoside, vitexin, vitexin-2"-O-glucoside, vitexin-2"-O-xyloside, isovitexin, schaftoside, isochaftoside) and luteolin (i.e. luteolin-6,8-di-C-glucoside, orientin, orientin-2"-O-rhamnoside,

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orientin-2''-*O*-xyloside, isoorientin) (Lutomski et al., 1981; Dhawan et al., 2004; Pereira et al., 2004; Zucolotto et al., 2011; Sakalem et al., 2012). Moreover, in extracts of *P. quadrangularis, P. alata, P. edulis* were found out saponins (Orsini et al., 1987; Reginatto et al., 2004; Yoshikawa et al., 2000; Birk et al., 2005; Sakalem et al., 2012) and harmala alkaloids but they may occur at low contents in various *Passiflora* species (*i.e. P. incarnata)* (Lutomski and Adamska, 1968; Lutomski and Nourcka, 1968; Lutomski and Malek, 1975; Grice et al., 2001). Besides, few in *Passiflora* species (*i.e. P. edulis, P. foetida, P. guatemalensis, P. morifolia, P. tripartita*) cyanogenic glycosides were marked (Jaroszewski et al., 1996; Andersen et al., 1998; Jaroszewski et al., 2002; Seigler et al., 2002; Saeki et al., 2011).

In modern European phytotherapy of sleep and anxiety disorders, the most valuable is *Passiflora incarnata* L. (EMEA 2008; Miroddi et al. 2013), which species is official in European Pharmacopoeia. In Germany and Poland the herb of this taxon *(Passiflorae herba*) has been used since the 1980s (Ozarowski, 1976; Deutsche Kommission E, 1990). According to Duke et al. (2009) *P. incarnata* shows many activities, *e.g.* adaptogenic, analgesic, antiaging, anti-inflammatory, antispasmodic, antistress, antitussive, and hypotensive. Similar effects mayalsobe exhibited by *P. edulis* Sims, *P. caerulea* L. and *P. quadrangularis* L. (de Castro et al., 2007; Duke et al., 2009; Sena et al., 2009; Deng et al., 2010; Feliú-Hemmelmann et al., 2013). Also it was shown that extract of *P. alata* and *P. incarnata* leaf exhibited the cytotoxic effect in acute lymphoblastic leukemia cell lines (CCRF-CEM) (Ozarowski et al., 2013a). At recent years, the fast fingerprint phytochemical analysis of methanolic extract of peel and juice-pulp of *P.tripartita* var. *mollissima* showed that they contain flavonoid *O*- and *C*-glycosides (Simirgiotis et al., 2013). Flavonoids were also observed in extract of *P. edulis* fruit pulp (Zeraik and Yariwake, 2010). This suggests that the juice and the maracujá" fruits can be considered as a functional food ingredient (Zeraik et al., 2010). ITI Tropicals Inc. according to (2013), Europeans have been using passion fruit (the main variety *Passiflora edulis* f. *flavicarpa* O. Deg.) for more than 30 years as an essential flavour in the food and beverage industry. Manufacturers in Europe have produced passion fruit based beverages and multi-vitamin drinks (iti Tropicals, 2012a), juice drinks, yogurt and tea (iTi Tropicals, 2012b). Therefore, high quality propagation materials of selected *Passiflora* plants rich in active metabolites could be produced only by asexual methods. Moreover, the conventional cultivation and diseases of *Passiflora* can seriously limit the productivity of all these species, especially in the moderate climateof Europe. In order to eliminate these difficulties, methods of plant *in vitro* culture can be applied as an alternative technique in pharmacognosy for the production of true-to-type plantlets from elite plants, which may contain valuable flavonoids. During the last decades, few reliable methods have been developed for micropropagation of *P. caerulea* (Vestri et al. 1990), *P. alata, P. mollissima, P. coccinea, P. herbertiana, P. suberosa* (Drew 1991), *P.amethystina* (Dornelas and Vieira, 1993), *P. incarnata* (Mingozzi et al., 2003). *P. cincinnata* (Dornelas and Vieira, 1993, Lombardi et al., 2007), *P. trifasciata, P. manta*, and *P. foetida* (Pipino et al. 2008). Except thatthe largest numberof earlierstudieswere carried outfor *P.edulis* and *P.edulis* f. *flavicarpa* (Dornelas and Vieira, 1993; Kawata et al., 1995; Biasi et al., 2000; Trevisan and Mendes, 2002; Winkler and Quoirin, 2002; Isutsa, 2004; Becerra et al., 2004; Davey et al., 2005).

This article is an overview of knowledge on the *in vitro* propagation of *Passiflora* species and presents data on various methods for their multiplication presented in the available literature between 2007 and 2013. The aim of this article is to show the main achievements and areaS for further research.

Traditional propagation

Conventional cultivation of *Passiflora* certain problems may pose concerning the percentage of seed germination, growth rate and viability of seedlings. Pires et al. (2012) found that for some species of *Passiflora* seedling emergence was observed until after 65-90 days of sowing seed. Additionally, it was noted that seeds of different *Passiflora* species have low rates of germination rate (Mendiondo and Garcia, 2006; Delanoy et al., 2006; Pires et al., 2012). Furthermore, microbiological contamination is a major challenge in the case of *Passiflora* species which secrete a sweet nectar and sap that are a nutrient medium for various microorganisms and sap-feeding insects. Plant diseases can seriously reduce the productivity of all *Passiflora* species. Pathogenic microorganisms which cause plant diseases are as follows: viruses (*Passion fruit woodiness virus, Passiflora latent virus, Passion fruit yellow mosaic virus, Purple granadilla mosaic virus*), bacteria (*Xanthomonas axonopodis pv. passiflorae, Pseudomonas syringae pv. passiflorae) and fungi (Fusarium solani, F. oxyporum* f. sp. *passiflorae, Cladosporium cladiosporioides, Alternaria passiflorae, Colletrotrichum gloeosporioides*) (Fischer and Rezende, 2008). They may produce compounds that are phytotoxic and/or a complex of enzymes that destroy the plant cell and tissue structures (Strange and Scott, 2005). Harmful to thegrowth of these plants are also the variety of insects (Balser, 2004). Due to the above problems, alternative ways for efficient methods of healthy plant propagation are needed.

Explants

In vitro studies were carried out on explants of *Passiflora* which originated from seedlings after germination *in vitro*, but in a few cases experiments explants were obtained from mature plants growing in a greenhouse (Becerraet al., 2004; Ozarowski et al. 2012; Pinto et al., 2010a, b; Ragavendran et al., 2012). Several authors have described *in vitro* plant regeneration via organogenesis by using nodal, internodal, leaf and hypocotyl segments. A few authors have observed somatic embryogenesis onmature zygotic embryos in *in vitro* cultures of *P. edulis, P. cincinnata,* and *P. foetida*. Moreover, micropropagation was performed using the shoot tips of *P. edulis, P. foetida,* and *P. incarnata* and transvers thin cell layer (TCL technology) (Nhut et al., 2007). All explants used to initiation of organogenesis were summarized at Chart 1.

Scientific research indicates that leaf fragments of *in vitro* plantlets were the most frequently used explants for induction of organogenesis. It is also important that orientation of leaf fragments placed on the basal medium

Chart 1
Commonly used growth regulators in various stages of in vitro propagation .

Species	Explant	Media + PGRs (μM)			Callus	Reference
		Organogenesis	Elongation	Rooting		
P. alata	ns, ins, ls	MSM + BA (4.4 - 22.0)	B5; ½ B5; ½ MSM; ½ MSM + 5% CW;½ MSM + 10% CW		(+)	Pacheco et al., 2012
P. alata	ls, hs	MS + TDZ (2.27); MS + BA (4.43); MS + BA (4.43) + TDZ (2.27); + $AgNO_3$	MSM + 10% CW or + GA_3 (2.88), MS + GA_3 (2.88)	½ MSM, ½ MS	(+)	Pinto et al., 2010a
P. caerulea	ls	MS+ BA (4.4)	nt	MS	(-)	Busilacchi et al., 2008
P. caerulea	ins	MS+ BA (4.4 or 8.87) + GA_3 (2.88)	nt	MS, ½ MS	(+)	Ozarowski et al., 2012
P. caerulea *P. incarnata*	st, ns	MS + BA (2.2 – 8.87), MS + IAA (0.57 – 5.7) + BA (2.2 – 8.87), MS + IAA (5.7) + BA (4.4) + GA_3 (2.88)	nt	MS, ½ MS, MS+ ½ agar	(+)	Ozarowski et al., 2013b
P. caerulea	rs	MS + 2.4-D (4.6 – 18.1)	nt	MS, ½ MS, MS+ ½ agar	(-)	Ozarowski et al., 2013b
P. caerulea	ls, ins, ps	MS + BA (2.2 – 8.87), MS + 2.4-D (4.6 – 18.1)	nt	MS, ½ MS	(+)	Ozarowski et al., 2013c
P. cincinnata	ze	Callus induction:MS + 2,4-D (4.6 – 27.1) + BA (4.5) Embryogenesis:MS + 2,4-D (4.6 – 27.1) + BA (4.5) + AC (3%)	MS + CA (3%)	nt	(+)	da Silva et al., 2009
P. cincinnata	ls, rs, s	MS + BA (2.2 – 8.87) + 5% CW	nt	nt	(+)	Lombardi et al., 2007
P. cincinnata	ze	MS + 2.4-D (18.1) + BA (4.5)	nt	nt	(-)	Rocha et al., 2012a
P. cincinnata	c	MS + 2.4-D (18.1) + BA (4.4)	nt	nt	(+)	da Silva, Carvalho, 2013
P. cincinnata	ze	Callus induction: MS + 2.4-D (18.1) + BA (4.4) Histodifferentiation: MS + AC (1.5%)	MS + GA_3 (1.45) + CA (1.5%)	sterile 3:1 $Plant_{max}$ + coconut	(+)	Pinto et al., 2010b
P. cincinnata	hs	MS + brassinosteroids (0.1 - 2.0 mg/l) + BA (4.4)	n.t.	nt	ni	da Silva et al., 2011a
P. cincinnata *P.edulis*	rs	MS + BA (4.4)	MS + GA_3 (2.89)	sterile 1:1 coconut fiber + $Plant_{max}$	(-)	da Silva et al., 2011b
P. edulis	ze	Callus induction: MS + 2.4-D (18.1 - 144.8), MS + 2.4-D (18.1 - 144.8) + BA (4.4), MS + BA (4.4). Histodifferentiation: MS + IAA (17), MS + IAA (17) + AC (1.5%), MS + IAA (17) + 2.4-D (72.4), MS + IAA (17) + 2.4-D (72.4) + AC (1.5%)	nt	nt	(+)	Pinto et al., 2011
P. cincinnata *P. edulis f. flavicarpa*	hs	MS + BA (2.2) + 10% CW	nt	nt	(+)	Dias et al., 2009
P. edulis	rs	MS + BA (4.4)	nt	nt	(-)	Rocha et al., 2012b
P. edulis f. *flavicarpa*	ls, hs	MS + BA (4.4) + 5% CW	nt	nt	(+)	Fernando et al., 2007
P. edulis, *P. edulis f. flavicarpa*	am	MS + BA (2.2 – 13.1)	nt	MS + IBA (0.98 – 5.90)	n.i.	Prammanee et al., 2011
P. edulis f. *flavicarpa*	ns	MS + BA (2.2 – 8.87), MS + KIN (2.3 – 9.3); MS + GA_3 (1.44 – 5.77); MS + BA (2.2 – 8.87) + KIN (2.3 – 9.3) + GA_3 (1.44 – 5.77)	½ MS + BA (22.2) + GA_3 (8.6 – 17.3)	½ MS, ½ MS + NAA (5.4), MS + NAA (2.7 – 10.7), or + IAA (5.7)	(+)	Nhut et al., 2007
P. hybrid (Guglielmo Betto)	at	Shoot induction: MS, MS + NAA (2.68) + 2iP (49.2), MS + NAA (11.4) + BA (4.4), Multiplication: MS + BA (0.02 - 0.18)	MS	MS	(+)	Pipino et al., 2010
P. foetida	st, ns	MS + BA (2.2 – 8.87), MS + BA (6.65) + KIN (2.3)	nt	½MS, MS + IBA (2.46 – 9.84)	(-)	Ragavendran et al., 2012
P. foetida	ze	MS + BA (4.5) + 2.4 D (13.5 – 27.1)	½ MS	nt	(+)	Rosa, Dorneas, 2012
P. foetida	ns, st, ins, ls	MS + BA (4.4 – 13.3); MS + BA (12; 13.3) + NAA (1.6); MS + BA (6.65; 8.87) + IBA (4.9)' MS + BA (6.65; 8.87) + IAA (5.7)	nt	MS + IBA (0.49 – 4.9) or IAA (0.57 – 5.7) or NAA (0.53 – 5.3)	(+)	Komanthi et al., 2011
P. gibertii	c	MS + PIC (0 and 2.07, 4.14, 6.21, 8.28) + KIN (0 and 0.46)	nt	nt	(+)	de Figueiredo Carvalho et al., 2013
P. setacea	ls, hs	MS + BA; MS + TDZ; MS + BA + TDZ	nt	nt	(+)	Vieira et al., 2011
P. suberosa	ins, ns, ls	MS/ MSM + BA (4.4 - 44.4), MS/ MSM + BA (4.4 - 44.4) + NAA (2.7, 5.4), MS + TDZ (4.54 - 45.4) or 2,4-D (4.5 - 45.2), or NAA (5.4 - 54), or PIC (4.14 - 41.4)	nt	½ MSM	(+)	Garcia et al., 2011

AC, activated charcoal;am, apical meristems; at, auxiliary tendrils; c, cotyledons;CW, coconut water;hs,hypocotyls segments; ins, internodal segments; ls, lea f segments; ns, nodal segments; ni, no information; nt, no transferred; ps, petiole segments; st, shoot tips; s, seedlings; ze, zygotic embryos.

can influence physiological and morphological responses of explants. According to Pacheco et al. (2012), direct shoot development was achieved when the abaxial surface of a leaf of *P. alata* was oriented upwards, while callus was produced in the opposite situation. Other authors have reported that organogenesis occurred in both orientations of leaf fragments of *P.alata* (Pinto et al., 2010a) and *P. cincinnata* (Lombardi et al., 2007). Moreover, it was shown that the age and physiological condition of thedonorplant are very important. Becerra et al. (2004) observed that among 1-6-month-old plants of *P. edulis* var. *flavicarpa*, only 2-month-old leaves showed the highest sensitivity response in plant *in vitro* culture. It follows that the regeneration capacity of explants was decreased with ageing of the mother plant. It is well known that young explant tissues constitute a more responsive for growth regulators. Therefore, seedlings of *Passiflora* are the most frequently used as a source of explants for initiation of *in vitro* studies.

Media composition and chemical additives

In all experiments Murashige and Skoog's medium supplemented with sucrose and agar was used. Besides plant growth regulators also medium composition and chemical additives such as silver nitrate ($AgNO_3$), coconut water (CW) or activated charcoal (AC) can influence the regenerative systems of *Passiflora*. Moreover, it was observed that for various stages of micropropagation not only MS medium was used but also B5, ½ B5, modified MS or ½ MS (Nhut et al., 2007; Pinto et al., 2010a; Garcia et al., 2011; Ozarowski et al., 2012, 2013b, 2013c; Pacheco et al., 2012; Ragavendran et al., 2012; Rosa and Dornelas, 2012; Rocha et al., 2012b). Pinto et al. (2010a) observed that direct organogenesis occurred more efficiently when explants of *P. alata* were cultured in media supplemented with $AgNO_3$ and cytokinins. Moreover, this result proved that silver nitrate is crucial for adventitious buds induction. The exact mechanism of action $AgNO_3$ on plant *in vitro* culture is unclear, but it was shown that this compound can probably antagonize ethylene action by reducing the receptor capacity to bind the gaseous signal molecule ethylene (Bleecker and Kende 2000; Kumar et al., 2009). Currently, scientific research indicates that silver nitrate has beneficial effects on regeneration and clonal propagation of several economically important plants (Kumar et al., 2009). According to a recent review on *Cocos nucifera* L. (Yong et al., 2009) are wide applications of CW observed. In recent years, a few studies have shown thepositive effect of CW in the *in vitro* cultures of *Passiflora* species. Basal medium enriched with CW (5-10%) was used for elongation of regenerated shoots of *P. alata* (Pacheco et al., 2012; Pinto et al., 2010a) and *P. cincinnata* (Lombardi et al., 2007), and for initiation *in vitro* organogenesis on explants of *P. edulis* f. *flavicarpa* (Fernando et al., 2007; Dias et al., 2009) and *P. cincinnata* (Lombardi et al., 2007; Dias et al., 2009). Moreover, it was observed that CW added to the basal medium significantly improved the root induction of *P. alata* shoots *in vitro* (Pacheco et al., 2012). Thus, CW increased the formation of adventitious buds, number of shoots per explant, shoot elongation rate, number of nodes per shoot and root development of *Passiflora* species.

It is well known that AC added to the basal medium can improve *in vitro* growth of plants by adsorbing toxic metabolites (Wang and Huang, 1976). Several medicinal plants are rich in phenolic compounds which can be secreted into the medium and may they have a toxic effect on the *in vitro* culture. Recent studies have shown that AC (3%) was used for induction of somatic embryogenesis and plant regeneration of *P. cincinnata* (da Silva et al., 2009; Pinto et al., 2010b), and for initiating the differentiation of embryogenic callus of *P. edulis* (Pinto et al., 2011).

All experiments were carried out on agar-gelled media and only two studies have been carried out using rotary liquid culture for regenerated shoot of *P. edulis* (da Silva et al., 2011b) and of *P. caerulea* (Ozarowski et al., 2013b).

Plant growth regulators

In most cases plant regeneration by organogenesis was induced using achieved BA with a wide range of concentrations. This cytokinin is essential for *in vitro* regeneration of *Passiflora* species, regardless of the type of explants, and the response to it varies with species and genotype (da Silva et al., 2011b). Cytokinins are very effective in initiation of direct or indirect shoot formation. Moreover, inducing the growth of adventitious shoots usually depends on the interaction between auxins and cytokinins. However, it is known that high concentrations of cytokinins are not always preferred in plant *in vitro* culture (George et al., 2008). In the studies of micropropagation of *Passiflora* taxa, cytokinins were used in a wide range from 2.2 to 44.4 µM of BA, with or without generally low concentrations of auxins (Chart 1).

In recent years several studies have aimed at developing theprocedures for micropropagation of *Passiflora* species (Chart 2). The highest regeneration efficiency was observed for nodal segments of *P. alata* but unfortunately organogenic explants formed calluses on medium supplemented with 13.2 µM BA (12.9 shoots per explant)(Pacheco et al., 2012). In another study the highest callus induction was observed from the shoot tip and node explants of *P. foetida* on MS medium supplemented with 13.2 µM BA and 2.7 µM of 1-naphthaleneacetic acid (NAA) and there were obtained from 13 to 17 on the regenerating shoots explant-derived callus (Komathi et al., 2011). Ragavendran et al. (2012) and Prammanee et al. (2011) reported that MS medium with BA (4.4 and 6.65 µM) was only the most effective for shoot formation on shoot tip culture of *P. foetida*, *P. edulis* and *P. edulis* f. *flavicarpa*. Prammanee et al. (2011) observed that shoot tips cultured with 6.65 µM BA generated many short shoots, whereas the tissue cultured with 4.4 µM BA generated long shoots. Nhut et al. (2007) and da Silva et al. (2011b) have also observed that lower concentration of BA (4.4 µM) was optimal for shoot regeneration of *P. edulis* and *P. cincinnata*. Moreover, *passion fruit woodiness virus*-free shoots were obtained. Ozarowski et al. (2009; 2013b; 2013c) studied the effect of cytokinins on *in vitro* shoot regeneration of *P. caerulea*.These authorsobserved that effective growth of adventitious shoots on the nodal explant of *P. caeruleaunder* the influence of 2.2 and 4.4 µM BA (max. 16 shoots). However, nodal tissue of *P. incarnata* cultured on MS

with 2.2 µMBA generated only a few short shoots (average 3.0 shoots/nodal fragment) (Ozarowski et al., 2013b). Pacheco et al. (2012) noted induction of direct organogenesis on internodal fragments of *P. alata* and 9.9 adventitious shoots per explant were obtained when BA was used in a concentration of 8.8 µM in modified MS (MSM) medium, whereas there was observed on leaf growth of only two shoots per explant on medium supplemented with 13.2 and 22 µM BA. Direct organogenesis was observed also on leaf and hypocotyl of *P. edulis* f. *flavicarpa* cultured on medium containing 4.4 µM BA (Lombardi et al., 2007). Garcia et al. (2011) observed the highest shoot regeneration from internodal segments of *P. suberosa* on MSM medium with very high concentration of 44.4 µM BA (12.79 shoots/explant). In addition, for leaf and nodal segments on MSM medium supplemented with a lower concentration of BA (22 µM), the production of 9.33 and 8.37 shoots/explant, respectively, was observed. Moreover, the MSM medium with BA was effective for both callus induction and shoot regeneration. Ozarowski et al. (2012) developed a rapid procedure for organogenesis on nodal segments of *P. caerulea* using 8.87 µM BA together with 2.88 µM of gibberellic acid (GA_3). Results showed the high shoot regeneration rate and bud forming capacity index and organogenic callus formation. Indirect organogenesis was observed on all kinds of explants of *P. suberosa* cultured on MS medium supplemented with BA and NAA. This combination of cytokinin and auxin resulted in the formation of shoots, although with reduced efficiency (Garcia et al., 2011). Komathi et al. (2011) also obtained the highest callus induction on MS with the same plant growth regulators for explants of *P. foetida*. Moreover, other phytohormones such as thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) used alone or in combination with BA induced callogenesis (Pinto et al., 2010a; Pinto et al., 2011; Rosa and Dornelas 2012; Garcia et al., 2011; Vieira et al., 2011; Ozarowski et al., 2013b; da Silva and Carvalho, 2013).

Da Silva et al. (2011b) established an efficient method for *P. edulis* and *P. cincinnata* regeneration using root fragments cultured on MS medium containing 4.4 µM BA and vitamins B5. In this study 42 shoots were obtained for both *P. cincinnata* and for *P. edulis* via thedirect pathway. The roots with developing shoots were transferred to MS liquid medium supplemented with 2.89 µM GA_3 for efficient shoot elongation. Previous studieshave shownalso shoot buds regeneration on root fragments of *P. cincinnata* under the influence of BA (2.2 and 5.87 µM) (Lombardi et al., 2007). Ozarowski et al. (2013b) also observed effective direct organogenesis on root fragments of *P. caerulea* in the rotary system of liquid MS medium with 18.1 µM 2.4-D.

In plant cultures *in vitro* somatic embryogenesis occurs most frequently as an alternative to the organogenesis for regeneration of whole plants and offers the possibility for large-scale clonal propagation (Kanwar and Kumar 2008). In recent years, few studies attempting somatic embryogenesis of *Passiflora* species have been reported (da Silva et al., 2009; Pinto et al., 2010b Pinto et al., 2011; Rocha et al., 2012a; Rosa and Dornelas 2012) (Charts 1 and 2).

Pinto et al. (2011) observed formation of embryogenic callus on mature zygotic embryo of *P. edulis* on MS medium including 2,4-D (18.1-144.8 µM) with or without BA (4.4 µM). Similar results were obtained for somatic embryogenesis of *P. cincinnata* but the larger number of somatic embryos was induced on medium with 2,4-D (144.8 µM) andBA (4.4 µM) (da Silva et al., 2009). Rocha et al. (2012a) and Pinto et al. (2010b) studied embryogenesis in only one MS medium supplemented by 2.4-D (18.1 µM) + BA (4.4 µM). Another study also showed that concentrations of 2,4-D (13.5 and 18 µM) with BA (4.5 µM) were most effective for plant regeneration from embryogenic callus of *P. foetida* (Rosa and Dornelas 2012). Authors of these studies described detailed anatomical, ultrastructural and biochemical alterations during somatic embryogenesis using light and scanning electron microscopy. It was shown that the primary embryogenesis pattern started in the region of the abaxial surface of the cotyledon and protuberances were formed from the meristematic proliferation of the epidermal and mesophyll cells for *P. cincinnata*. The large nuclei, dense cytoplasm with a predominance of mitochondria, and a few reserve compounds were observed (Rocha et al., 2012a). Authors that showed the conversion of well-formed somatic embryos to plants in MS-based medium lacking growth regulators was achieved the high frequencies with the 60%, and the plants were successfully acclimatized (da Silva et al., 2009). Moreover, it was exerted that 2.4-D and BA are key factors determining the embryogenic response not only for various *Passiflora* but also for several other species (da Silva et al., 2009).

Root development, acclimatization and field establishment

Recent analysis of various studies has shown that effective rooting of regenerated plants was observed on basal medium with full and half strength MS without supplementation of plant growth regulators (Becerra et al., 2004; Pinto et al., 2010a; Garcia et al., 2011; Ozarowski et al., 2012, 2013a, 2013b; Pacheco et al., 2012). Furthermore, Ragavendran et al. (2012) and Prammanee et al. (2011) used MS medium supplemented with indole-3-butyric acid (IBA) for *P. foetidaand P. edulis*. Other authors observed that vigorous rooting of *P. edulis* f. *flavicarpa* plantletson ½ MS medium with indole-3-acetyl-L-aspartic acid (IAA) (Nhut et al., 2007). Root development (70-100%) was observed usually after 30 days of culture (Pinto et al., 2010a; Komathi et al., 2011; da Silva et al., 2011b; Garcia et al., 2011; Ozarowski et al., 2012; Pacheco et al., 2012). On the basis of these results it can be concluded that auxins are not necessary for rooting shoots of *Passiflora*. *In vitro* regenerated plants were successfully (100%) acclimatized to green house conditions (da Silva et al., 2009; da Silva et al., 2011b) (Chart 2).

Genetic stability and phytochemical profile of regenerated plants

The use of *in vitro* cultures should be performed in special conditions to avoid changes in the plant genome. Especially, which outgrowth from meristems are formed again (adventitiously) from explants or callus may show genetic disturbances, which result in somaclonal variation. Confirmation of genetic stability during micropropagation is

Chart 2
Summary of work on organogenesis and embryogenesis.

Species	Range of research	Main response		Reference
		Organogenesis	Elongation (E)/ rooting (R)/ acclimatization (A)	
P. alata	Direct and indirect organogenesis; cell suspension cultures	12.9 shoots/ns (MSM + 13.2 µM BA); 9.9 shoots/ins (MSM + 8.8 µM BA); 2 shoots/ls (MSM + 13.2 µM BA)	E - ½ MSM + 10% CW R - 100% on MSM + CW A - ni	Pacheco et al., 2012
P. alata	Organogenesis; microscopical analysis	The best results on media with AgNO3. Higher number of responsive explants (ls) on MS + TDZ + AgNO3 (58%), on MS + BAP + TDZ + AgNO3 (44.4%)	E - MSM + GA_3 (35% of total shoot buds) R - auxins not necessary A - ni	Pinto et al., 2010a
P. caerulea	Direct organogenesis; histological studies; TLC chromatography	Adventitious buds originated from the proliferation tissue mass without previous callus formation. 70% of the explants regenerated shoots. Similar chromatograms for extracts obtained from mother plants and plantlets.	E – ni R – MS A – 100%	Busilacchi et al., 2008
P. caerulea	Direct and indirect organogenesis; HPLC and HPTLC phytochemical analysis of callus	Callus induction (100%) on MS+BA+GA_3 and MS+2.4-D. On MS+ BA (4.4 µM) + GA_3 (2.88 µM) – highest shoot regeneration rate (106.4%), bud forming capacity index (3.1) for stem-derived callus. HPLC analysis: vitexin, isovitexin and rutin, chlorogenic acid, rosmarinic acid inlow concentrations.	ni	Ozarowski et al., 2012
P. caerulea *P. incarnata*	Direct and indirect organogenesis; HPLC and HPTLC phytochemical analysis of plantlets	16 shoots/ ns (+callus) on MS + BA (4.4 µM) - *P. caerulea*, 3 shoots/ns on MS + BA (2.2-4.4 µM) - *P. incarnata*, but effective regenerative response of lateral meristems (100%) on MS without any plant growth regulators. HPLC analysis: similar phytochemical profile of plantlets and mother plants, but extracts of plantlets contained higher concentrations of phenolic compounds.	E - ni R - MS, ½ MS, MS + ½ agar A - 100%	Ozarowski et al., 2013a
P. caerulea	Direct organogenesis	3 shoots/r.s on MS + 2.4-D (18.1 µM) in rotary system of liquid medium (effectiveness 90%)	E - ni R - 100% on MS A - 100%	Ozarowski et al., 2013a
P. caerulea	Direct and indirect organogenesis, HPLC phytochemical analysis of plantlets	Morphogenic and non-morphogenic callus on MS + BA and MS + 2.4-D, respectively. The highest of bud forming capacity for leaf-derived callus on MS + BA (8.8 µM), for petiole-derived callus on MS + BA (4.4 µM). 3 shoots (3 cm)/explants on MS + BA (8.8 µM). HPLC analysis (MS + BA 8.8 µM): isovitexin > chlorogenic acid > rutin > hyperoside > vitexin > luteolin > apigenin > rosmarinic acid and no alkaloids.	E - ni R - 100% on MS A - ni	Ozarowski et al., 2013b
P. cincinnata	Somatic embryogenesis	The largest number of somatic embryos from calli on MS + 2,4-D (18.1 µM) + BA (4.5 µM), Some abnormalities: fused axes, fused cotyledons and polycotyledonary embryos.	E - 60% on MS + CA R – ni A – ni	da Silva et al., 2009
P. cincinnata	Direct and indirect organogenesis; anatomical studies	76% of explants formed buds on rs (4.4 µM, 5.87 µM BA); 54% of explants formed buds on ls (MS + 2.2 µM BA); 3.9 shoot/ls (MS + 2.2 µM BA); 5.1 shoot/rs (MS + 2.2 µM BA)	ni	Lombardi et al., 2007
P. cincinnata	Somatic embryogenesis; Histocytological, histochemical evidences	Differentiation of the somatic embryos in the abaxial side of the cotyledon region and protuberances from the meristematic proliferation of epidermal and mesophyl cells.	ni	Rocha et al., 2012a
P. cincinnata	Somatic mbryogenesis;flow cytometric analysis	Multiple somatic embryos on cotyledonary leaves after 90 days of cultivation in induction medium. DNA ploidy level: up to 8C. Somaclonal variation.	ni	Silva, Carvalho, 2013
P. cincinnata	Somatic embryogenesis; flow cytometric analysis	Regenerated plants from embryogenic calli maintained true-to-type ploidy. From the 100 zygotic embryos obtained 305 normal plantlets.	E – ni R – ni A - 90%	Pinto et al., 2010b

Chart 2
(Continued).

P. cincinnata *P. edulis*	Organogenesis, anatomical and ultrastructural analysis; flow cytometric analysis	42.40 shoots/rs (*P. edulis* FB 200 and *P. cincinnata*) after 90 days. Any variations in DNA content of regenerated plantlets.	E - MS + GA_3 (liquid medium) R - 80% A - effective	da Silva et al., 2011b
P.edulis	Somatic embryogenesis; cytological, histological analyses	The highest frequencies of embryogenic calli on 2,4-D (72.4 µM) + BA (4.4 µM). Efficient histodifferentiation on MS + CA + IAA with/without 2,4-D.	No conversion of somatic embryos into plantlets	Pinto et al., 2011
P. cincinnata *P. edulis f. flavicarpa*	Organogenesis; polyamine, ethylene measurements;anatomical studies	Organogenesis on the peripheral areas of explants with vascular connections established between the callus and developing buds and shoots. Morphogenic responses with production of elevated levels of polyamine and ethylene.	ni	Dias et al., 2009
P. edulis	Organogenesis; anatomical and ultrastructural analysis	Adventitious buds and nodules formed from meristemoids on root segments via direct and indirect organogenic, originated from the pericycle regions distant from the cut surface. Differentiated buds - after 20 days of culture.	ni	Rocha et al., 2012b
P. edulis f. flavicarpa	Direct and indirect organogenesis; ultrastructural analysis	23 buds/48 ls, 150 buds/48 hs. Both direct and indirect regeneration modes on hypocotyl explants, but only direct regeneration occurred in leaf-derived cultures.	ni	Fernando et al., 2007
P. edulis, P. edulis f. flavicarpa	Plant regeneration from the meristem tip of virus-infected plant, ELISA technique.	Successfully regenerated of virus-free plants. On MS + BA (4.4 and 6.65 µM) greater number of shoots.	E - ni R - MS + IBA (1.96, 2.95µM) A - ni	Prammanee et al., 2011
P. edulis f. flavicarpa	Protocol for micropropagation; ransverse thin cell layer (tTCL)method	Optimal for shoot regeneration – MS + 4.4 µM BA; 100% explants regenerated shoots. Callus formation in all cases.	E - ½ MS + BA (22.2 µM) + GA_3(17.3 µM) R - vigorous on MS + IAA (5.7 µM); A - ni	Nhut et al., 2007
P. hybrid (Guglielmo Betto)	Protocol for micropropagation	Initiation: 11.2 shoots/at on MS + 2iP (49.2 µM) + NAA (2.68 µM). Multiplication: 8.5 shoots/explant on MS + BA (0.11 µM)	E, R - MS A - 70%	Pipino et al., 2010
P. foetida	Protocol for micropropagation	3.6 shoots/explants on MS + BA (6.65 µM); 3.1 shoots/explants on MS + BA (8.87 µM)	E - MS + BA (6.65µM) R - 90% on MS + IBA (4.9µM) A - 78%	Ragavendran et al., 2012
P. foetida	Organogenesis; anatomical and ultrastructural analysis; studies of the process of secretory trichome differentiation	34.9 shoots/callus on MS +2,4-D (13.6 µM) + BA (4.5 µM); 15 shoots/callus on MS + 2,4-D (18.1 µM) + BA (4.5 µM); 15 of trichomes per regenerated leaf on MS + 2,4-D (18.1 µM) + BA (4.5 µM)	ni	Rosa, Dorneas, 2012
P. foetida	Protocol for micropropagation	13 shoots/callus from ns on MS + BA (13.3 µM) + NAA (1.6 µM) and on MS + BA (6.65 µM) + IAA (5.7 µM) 17 shoots/callus from st on MS + BA (13.3 µM)	E - MS + BA (8.87 µM) + IAA (5.7 µM); R - 70%, 20 roots/shoot on MS + IBA (4.9 µM) A - 75%	Komanthi et al., 2011
P. gibertii	Organogenesis; embryogenesis; ultrastructural analyses	MS + 4.14 µM PIC + 0.46 µM KIN - the most suitable to induce embryogenic cells. Detailed structural information about the embryogenic callus culture	ni	de Figueiredo Carvalho et al., 2013
P. setacea	Direct and indirectogranogenesis	Regenerated shoots at light and darkness on MS + BA + TDZ	ni	Vieira et al., 2011
P. suberosa	Callogenesis; direct and indirectogranogenesis	12.79 shoots/ins on MSM + BA (44.4 µM) 9.33 shoots/ls on MSM + BA (22.2µM) 8.37 shoots/ns on MSM + BA (22.2µM) Non-morphogenic calluses on MS +TDZ, PIC, 2,4-D, and NAA.	E – ni R - 100% on ½ MSM A - 100%	Garcia et al., 2011

of particular importance in medicinal plants for production of certified plant materials to obtain herbal medicines. Also, the presence and the composition of these secondary metabolites should remain unchanged after micropropagation (Sliwinska and Thiem, 2007, Thiem and Kikowska, 2008). Nonetheless, some authors have not only studied micropropagated plants of *P. edulis* and *P. cincinnata* in MS with BA (2.2 µM) and then with GA_3 (2.89 µM) (da Silva et al., 2011b), but also embryogenic callus culture of *Passiflora cincinnata* in MS supplemented with BA (4.4 µM) and 2,4-D (18.1 µM) (da Silva and Carvalho, 2013; Pinto et al., 2010b). The identification of somaclonal variation was performed by flow cytometry (FCM) to determinaine of DNA ploidy level. Results showed that prolonged cultivation in medium containing 2,4-D influenced on higher DNA ploidy levels in callus cells. Thus, it was concluded that in order to prevent the emergence of undesired during ploidies clonal propagation, embryogenic callus culture time should not be prolonged (da Silva and Carvalho, 2013). Moreover, Pinto et al. (2010b) evaluated 100 somatic embryogenesis-derived *P. cincinnata* plants and one plant regenerated showed double DNA content. Da Silva et al. (2011b) observed no variation in the DNA content of regenerated plantlets of *P. cinncinata* and *P. edulis*.

On the other hand, there is a lack of systematic phytochemical evaluation for *in vitro* clones for the detection of flavonoids, phenolic acids and alkaloids in regenerated plantlets of *Passiflora*. To date, only Busilacchi et al. (2008) and Ozarowski et al. (2012, 2013b, 2013c) have confirmed the occurrence of secondary metabolites in plantlets by chromatographic methods. HPTLC and HPLC analysis of methanol extracts of regenerated plants PC and PI on MS medium with BA (8.8 µM) showed presence of apigenin, luteolin, vitexin, isovitexin, rutin, hyperoside, chlorogenic and rosmarinic acids (Ozarowski et al., 2013b, Ozarowski et al., 2013c) (Chart 2). Phytochemical studies, mainly HPLC-MS analysis, are in progress.

Conclusion and future considerations

Medicinal plant propagation *in vitro* has been shown to be feasible for commercial production of elite plants of *Passiflora*. The review showed that an organogenesis-based plant regeneration system using 6-benzyladenine is currently prevailing in species of *Passiflora*, because direct and indirect morphogenesis are frequently occurring processes for these plants. Moreover, silver nitrate, coconut water or activated charcoal added to basal medium exerted a beneficial effect on regenerative systems. In the other hand, it should be noted that there are difficulties in comparison due to the lack of comparable parameters studies, because not all researchers performed the same experiment, some focused only on selected physiological aspects. In the future there is a need to conduct a full protocols that will take into account all stages of micropropagation as the establishment of primary cultures, shoot multiplication, rooting of regenerated plants and acclimatization. Moreover, the processes of the micropropagation based on organogenic callus still need to be improved. Importantly, reproducible protocols including somatic embryogenesis may open novel regeneration system for mass propagation of *Passiflora* species (da Silva et al., 2009). Moreover, it seems that methods as TCL and micropropagation in bioreactors may be used for optimization of mass propagation of healthy regenerated plants (Ziv, 2000, da Silva 2003, da Silva et al. 2007, Nuth et al., 2007). The plant regeneration in the liquid medium may be easier than on a solid medium (Ziv 2005, Yesil-Celiktas et al., 2010). According to Pack et al. (2005) automation of micropropagation via organogenesis or somatic embryogenesis in the bioreactors has been advanced as a possible way of reducing costs, *i.e.* by using the temporary immersion system automated the industrial future method for clonal propagation of *Passiflora* species.

In summary, shoot cultures and plantlets of *Passiflora* species should be evaluated morphologically, cytogenetically, physiologically, biochemically and phytochemically. According to modern standards, the microbiological quality of micropropagated plants is also necessary. It seems that more systematic studies are needed in order to obtain the valuable biomaterial and to explain the influence of the bio-physical-chemical conditions on induction, biomass growth and secondary metabolites synthesis in plant *in vitro* cultures obtained from different explants of *Passiflora* sp.

Authors contributions

MO contributed in the systematic review of the scientific bibliography, analysis and classification of available results of research on micropropagation using various technique of plant *in vitro* culture and phytochemical profile and main pharmacological activities of representative plants from genus *Passiflora*. Key steps in the systematic review were: (1) formulation of a focused review question, (2) a comprehensive and exhaustive search with inclusion of primary study and selection of investigations, (3) quality assessment of included studies and data extraction, (4) synthesis result of study, (5) interpretation and comparison of results. Literature searches were using performed the following databases: SCOPUS, PubMed and Natural Medicines Comprehensive Database (NMCD), European Scientific Cooperative on Phytotherapy (ESCOP) and European Medicines Agency (EMA) monographs. In the methodology used MO following criteria for systematic review: key words, time of publication date, thekind of investigation and availabilityof publications. MO wrote andedited themanuscriptand hasa correspondingauthor. BT contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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