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ORIGINAL ARTICLE

Long-term in vitro culture affects phenotypic plasticity of *Neoregelia johannis* plants

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Abstract

Factors such as stress and prolonged in vitro culture may lead microplants to senescence, reducing and/or altering their morphogenic potential. Considering that plants phenotypic plasticity is improved under stress, this research investigated this physiological event in *Neoregelia johannis* microplants in long-term in vitro culture. Histological and histochemical analyses were done to monitor the morphogenic potential during the propagules induction by microplants in vitro for 1 and 5 years, and from young leaf used as explants from five subcultures of microplants in both origins. During the monitor of morphogenic pathways, have not been seen alteration for the axillary meristems establishment and development besides that, the microplants multiplication have shown parenchyma cells in the proximal and in shoot bases acting as a pluripotent cell niche to direct adventitious organogenesis (adventitious buds) for young and older cultures. These events were evidenced by amyloplasts and polysaccharides in shoot bases and its reduction along the subcultures to induce adventitious and axillary buds. The culture medium renewal induced stress in microplants as well as the probable aging in older microplants, and it conducted the epidermal and subepidermal cells of older leaves to act as target cells niches for pluripotency and totipotency from the second subculture of leaf explants. All the adventitious roots were originated directly from the meristematic activity of procambial cells and older microplants roots acquired competence to indirect somatic embryogenesis after the second foliar subculture. Therefore, our results indicates that long-term in vitro culture supports new morphogenic competence in microplants, resulting from cell and phenotypic plasticity, that could help in the species survival.

Key message

Long-term in vitro culture acquired in microplants new morphogenic competences due to the stress caused by the culture medium renewal and its probable aging, resulting to cell and phenotypic plasticity.

Keywords *Neoregelia johannis* · Micropropagation · Stress · Aging · Pluripotency · Totipotency

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Abbreviations

NAA 1-Naphthaleneacetic acid
BAP 6-Benzylaminopurine
MS Murashige and Skoog culture medium

Introduction

An organism can have distinct phenotypes and it may originate in response to environmental variations, which is named as "truly" phenotypic plasticity. When the variation patterns follows up allometric pathway, and could result in plant size variation, it is named as "apparent" phenotypic plasticity (DeWitt et al. 1998; McConnaughay and Coleman 1999; Wright and McConnaughay 2002; Weiner 2004; Chambel

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et al. 2005; Barberis et al. 2017). However, it is likely that a temporal variation improves the plasticity in order to spatial variation; because it supports the phenotypic variability, while the spatial variation supports a discretely fixed phenotype (Scheiner and Holt 2012). Furthermore, the stem cell immortality, the vascular self-determination, and its epicormic branching are important characteristics for plants phenotypic plasticity, contributing to their longevity (Borges 2009). Differentiated cells can become undifferentiated, acquiring new cells fates. It has been known as cell plasticity and commonly occur when the plant tissue culture technique is used (Díaz-Sala 2014; Almeida and Almeida 2006; Almeida et al. 2012, 2015). Thus, micropropagation can lead to obtaining large-scale plants (Phillips 2004; Almeida et al. 2012, 2015) by multicellular organism development as well its organs and tissues differentiation (Zhuravlev and Omelko 2008; Almeida et al. 2012, 2015). This process has been known as morphogenesis (Taylor 1997; Almeida et al. 2015), and is mainly controlled by a signal transcription network and plant hormones (Smet et al. 2009; Almeida et al. 2012, 2015). However, for some species, the long-term in vitro culture can lead to indirect pathway (callus) and it have been reported as inducing changes in the morphogenic potential (Chaturvedi and Mitra 1975; Murashige and Nakano 1965; Van 1981; Konan et al. 2010; Graner et al. 2018), reducing root system development (Sharma et al. 2007), the survival rate of acclimatization (Konan et al. 2010), which it is probably due to clones senescence (Häsler et al. 2003; Konan et al. 2010) and the plant aging (Valledor et al. 2007). Nevertheless, the consequences of a long-term in vitro culture in species that have been obtained with direct regeneration (without previous callus induction), especially in perennial species, has not been found in the current literature.

The chronological aging follows the lifetime of a single plant, and it can be by germination or propagation (clones), although an aged plant can return to its vigorous and productive stage by the use of various horticultural practices (Valledor et al. 2010; Wendling et al. 2014a). The aging on plants cultivated in vitro, which were obtained by the indirect pathway (callus) have been shown a decrease in its morphogenetic potential (Chaturvedi and Mitra 1975; Murashige and Nakano 1965; Van 1981; Konan et al. 2010; Graner et al. 2018), and for the direct pathway (free of callus structures), has already been reported by Graner et al. (2018). Graner et al. (2015) had reported that peach palm long-term in vitro culture caused generalized senescence (programmed cell death) in foliar tissue cells, on the stem bases with the apical meristem and in some roots, it could evidence the probable plant aging, however, without causing the death of the microplants.

The senescence process is commonly defined as a part of the plant aging, and can occur during advanced stages in an organism development and it is regulated by genes related to the nutrients remobilization, culminating with the cell, tissue, organ or plant death (Graner et al. 2015). The senescence occurs during all plants life, reaching its climax with cell death during its aging process. Thus, the aging refers to some changes that occur over time, not resulting in the organism death, while senescence culminates in the death of certain cells, tissues or organs (Medawar 1957).

Therefore, to confirm that culture in vitro for 5 years promotes aging and its subsequent cellular and phenotypic plasticity in *Neoregelia johannis*, histological and histochemical analyses were performed in stem bases longitudinal sections, and compared to the same done in younger microplants of this specie, with 1 year in vitro.

Materials and methods

Plant material

To identify plants phenotypic plasticity, we used *N. johannis* (Carriére) L.B. Smith (*Bromeliaceae*), that were established and maintained in vitro for 1 and 5 years-old from the establishment of two clonal microgardens, according to Meneghetti (2015) (Fig. 1).

Culture conditions

Plants in vitro for 1 and 5 years-old

The seedlings were obtained from germinated *N. johannis* in vitro in Murashige and Skoog (1962) (basic MS culture medium), without plant hormones and pH adjusted to 5.8. Subsequently, the seedlings were transferred for the same basic culture medium supplemented with 0.27 µM of NAA (1-Naphthaleneacetic acid, Sigma®-Aldrich, catalog number: N0640) and 2.22 µM of BAP (6-Benzylaminopurine, Sigma®-Aldrich, catalog number: B3408) (culture medium for multiplication) for 3 months. A single microstump were isolated from a seedling, and its sprouts (adventitious buds) were individualized and transferred for the multiplication culture medium cited above and then they were cultivated for more 3 months, thus forming a clonal microgarden that was created by Meneghetti (2015) (Fig. 1). The microplants were individualized and subcultured every 30 days to maintain its quality and freshness in the basic culture medium for 3 months (microplants elongation), intercalating between culture medium for multiplication (3 months, with culture medium renewal every 30 days), for 5 years (long-term culture in vitro) (Fig. 1). The cultures were maintained in glass pots with plastic lids (7.0 cm in diameter and 8.0 cm in length), with culture medium supplemented with 6.0 g/L of agar (Sigma®-Aldrich, catalog number:

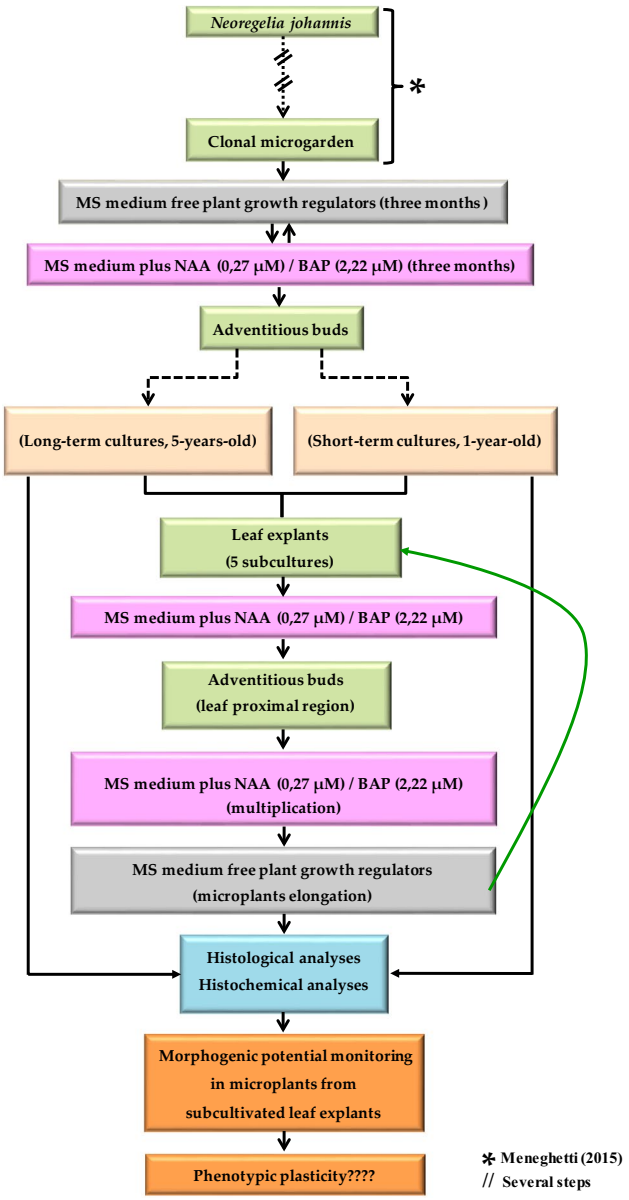

Fig. 1 In vitro culture and analyses of *N. johannis* microplants

A-7921). The medium was renewed every 30 days to maintain its quality and freshness, returning the cultures to the respective culture medium (microplants elongation or multiplication).

The second batch of seedlings was obtained from the *N. johannis* germinated in vitro with the same culture conditions described above for the seedlings, to obtain a new clonal microgarden and microplants were established and maintained in vitro by 1 year (Fig. 1).

All the cultures were maintained in a growth room at 25 ± 2 °C under a light intensity of 42 µmol m^{-2} s^{-1} for a 16 h photoperiod.

Microplants obtained from leaf explants

Microplants with 1 and 5 years-old were used for the explants collect that measured between 5.0 and 6.0 cm of length (stem base at the apex of the elongated leaves).

Explants from the two first fully expanded leaves (2.0–2.5 cm of length) in the apex-base direction of the first and second leaflets in microplants from both origins, at 1 and 5 years age were established in vitro and maintained in medium MS at Pyrex culture tube closed with Kim Kap 73660-25 plastic lids, without gelling agents and supplemented with NAA (0.27 µM) and BAP (2.22 µM) (multiplication culture medium) for shoots inducting and development, its pH was adjusted to 5.8 (Meneghetti 2015) (Figs. 1, 2). The adventitious buds were individualized, multiplied and elongated in culture medium with 6.0 g/L of agar (Sigma®-Aldrich, catalog number: A-7921) in glass pots with plastic lids with 7.0 cm in diameter and 8.0 cm in length to collect new leaf explants, making five successive subcultures (Figs. 1, 2). One hundred and fifty leaf explants were used for each subculture and inoculated into test tubes containing three to five explants.

The cultures were maintained in controlled growth room at 25 ± 2 °C under a light intensity of 42 µmol m^{-2} s^{-1} for a 16 h photoperiod and were sub cultured every 30 days for medium renewal.

Histological and histochemical analyses

Ten whole microplants from the *N. johannis* clones (5-year-old: long-term in vitro culture, one-year-old: short-term in vitro culture, and microplants obtained by the five subcultives of leaf explants) were submitted to histological and histochemical analyses (Fig. 1). To elucidate the somatic embryos origin that we observed in older microplants roots, ten samples containing somatic embryos were also submitted to histological and histochemical analyses.

The samples were fixed in a glutaraldehyde and formaldehyde solution (Karnovsky 1965) and were dehydrated through a graded alcohol series [10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% ($v.v^{-1}$)]; incubated in each alcohol concentration for 10 min. Finally, the samples were embedded in hydroxyethyl methacrylate resin (Leica, Heidelberg, Germany), according to the recommendations of the manufacturer and were cut, using a rotary microtome, (E. Leitz, Wetzlar, Germany) into 5 µm longitudinal sections. Next, the samples were submitted to specific histochemical tests to verify polyphenols, proteins, and polysaccharides presence during morphogenic events with periodic acid-Schiff stain and naphthol blue–black (Fisher 1968) as previously described by Almeida et al. (2012). As a result, the polysaccharides in the cell wall, cytoplasm, and the amyloplasts were identified by their

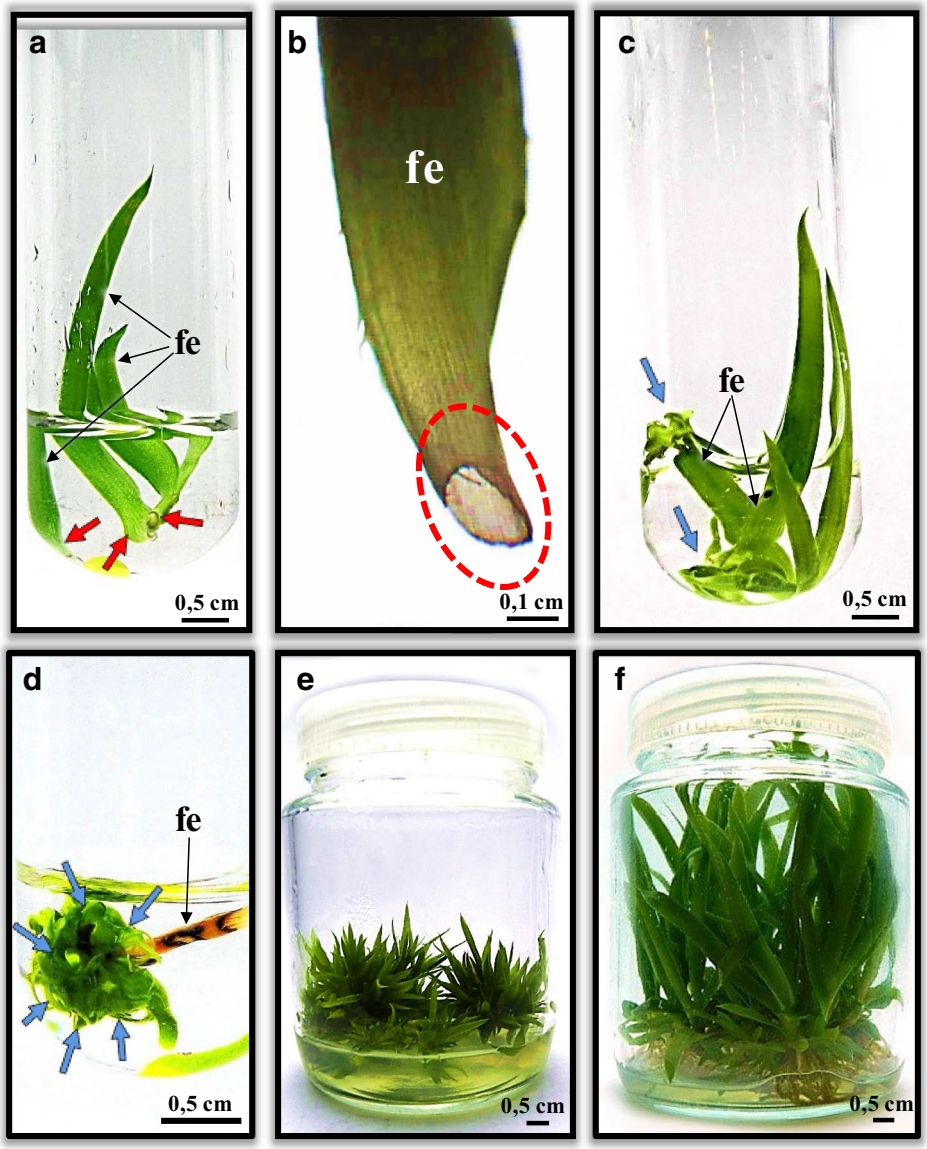

Fig. 2 Microplants were obtained from five subcultures successive of *N. johannis* leaf explants and were maintained in vitro for 5 years and for 1 year. **a** Leaf explants in MS medium for multiplication. **b** Detail of the proximal region in a foliar explant. **c**, **d** Adventitious buds induced in the proximal region of leaf explants. **e** Sprouts obtained in ***a***, ***c***, ***d*** in MS medium for multiplication. **f** Microplants in MS medium free plant growth regulators (microplants elongation). *fe* Foliar explant, *blue arrows* cluster of adventitious buds induced in the proximal region, *red arrows* and *red dashed line* proximal region of leaf explants. (Color figure online)

pink color, while the phenolic compounds were labeled with the orange color from the periodic acid-Schiff. Proteins were stained blue by the naphthol blue–black. All the histological sections were analyzed and photomicrographed under a light microscope (Carl Zeiss-Jenemed2, Oberkochen, Germany) equipped with a Samsung camera (SDC-313 Series, Samsung Techwin Co., Ltd., Changwon city, Korea).

Analysis and interpretation data

The histological and histochemical analyses presented a descriptive character from the interpretation of the glass microscope slide.

Results and discussion

Histological and histochemical analyzes were carried out in this research aiming to prove the possible occurrence of cellular and phenotypic plasticity in long-term in vitro culture microplants, after five successive subcultures of leaf explants (Figs. 1, 2, 3). The Fig. 3 shows that the propagules leaf explants induction from older microplants increased considerably until the third subculture in vitro, when we compared with the obtained data from the younger microplants leaf explants. On this moment, the number of explants from both experiments didn't presented alteration until the end of the fifth subculture (end of experiment). These data could be associated with

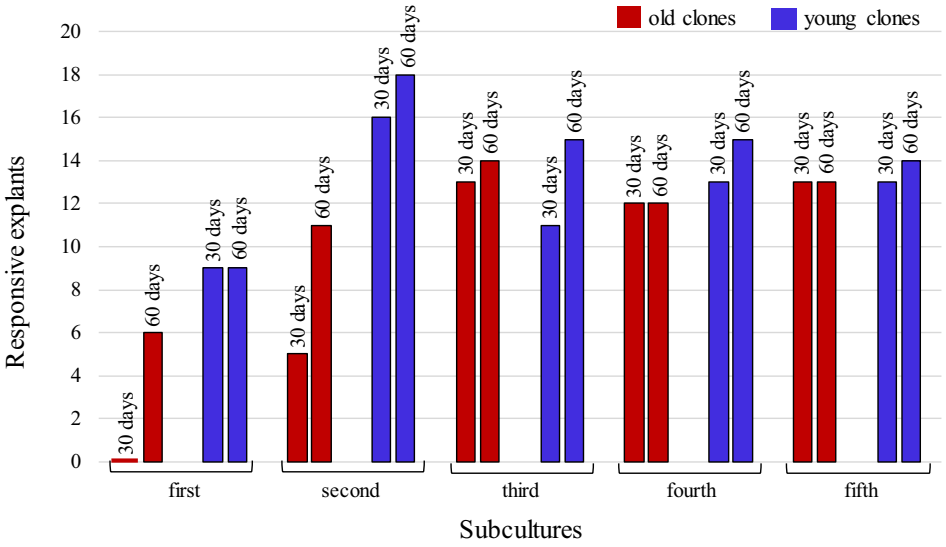

Fig. 3 Responsive leaf explants after 30 and 60 days of inoculation at the first to the fifth subculture. (Color figure online)

a possible rejuvenation of the plant or even to a better vigor (Wendling et al. 2014a, b) that was observed in older microplants until the third subculture of leaf explants. This response by the increase of the explants number in older microplants constitutes a continuous supplying of explants for the establishment of a microclonal garden.

In typical bromeliads, axillary buds occur throughout the shoot rosette extension, as well as on the basal region, giving rise to new shoots, however, some saxicultural species, which have a longer life cycle, such as *Pitcairnia* spp., can lose the shoots induction competence according to its physiological status or some external factors, such as photoperiod (Benzing 2000). Most likely, the axillary meristems post-embryonic origin from cells separated from the shoot apical meristem, simultaneously to the initiation of the foliar primordia, which maintain the morphogenic potential and with acropetal development (from base to apex), through specific environmental and endogenous stimuli (DiDonato et al. 2004; Beveridge et al. 2007; Rodrigues and Kerbauy 2009).

The adventitious buds evidenced an acropetal development in both older and younger microplants, as well as those from successive subcultures of leaf explants (Fig. 4a–l). It was confirmed by the energy resources content (amyloplasts and polysaccharides) in stem bases proximal and distal regions and a reduce of these substances along the microplants subcultures due to the high requirement for the axillary buds resumption activity in the proximal bases and the adventitious buds induction at the proximal end during cultivation in culture medium for multiplication (Figs. 5a–f, 6a–f, 7a–c). Both ages of the microplants multiplication were analyzed and, we observed that the parenchyma cells of the stem base proximal end act as a pluripotent cell niche to direct adventitious organogenesis (adventitious buds) (Fig. 7a–c). We also observed a higher morphogenic potential in the older microplants, from the second foliar explants subculture, since adventitious buds (adventitious organogenesis) also was observed by the prior protuberance induction, in the older leaves in the proximal region of these microplants maintained in vitro for 5 years by prior induction of a protuberance (Fig. 8a–f). Similar result was observed by Meneghetti (2015) and Alves et al. (2006) in histological and histochemical analysis carried out on the first two totally expanded leaves in the apex-base direction of microplants how described for this species. These protuberances were developed in leaf explants proximal region, from the periclinal and anticlinal divisions in mesophyll parenchyma cells (Meneghetti 2015) and caused to the separation of these leaves from maternal tissue (Fig. 8a–l). Most likely, the development of these protuberances in the proximal region were favored by the older leaves contact with the culture medium supplemented with plant hormones, which could subsequently have been induced to an oxidative process that evidenced the cells death in tissues of this region (Ferreira 2009) (Fig. 8e, f). The cell death process induction in these protuberances favored the nutrients allocation for the meristematic activity destined to the organogenesis, which was observed (Fig. 8a, e, f) (Almeida et al. 2012; Joy et al. 1991). The deformed cells, with cell wall degeneration, plasma membrane retraction, the presence of condensed nuclei and degenerative processes; as well as the high phenolic compounds content due to oxidation and cell walls degeneration, are cell death process characteristics (Graner et al. 2015) (Fig. 8e, f). Thus, mesophilic parenchyma cells acted as a pluripotent cell niche for the direct induction of adventitious buds, which often had large axillary bud sizes and buds in the proximal region (Fig. 8a–f).

In the older leaves median third, the parenchyma cells acted as competent cells for somatic embryogenesis (Fig. 8g–i), and they were characterized by the occurrence

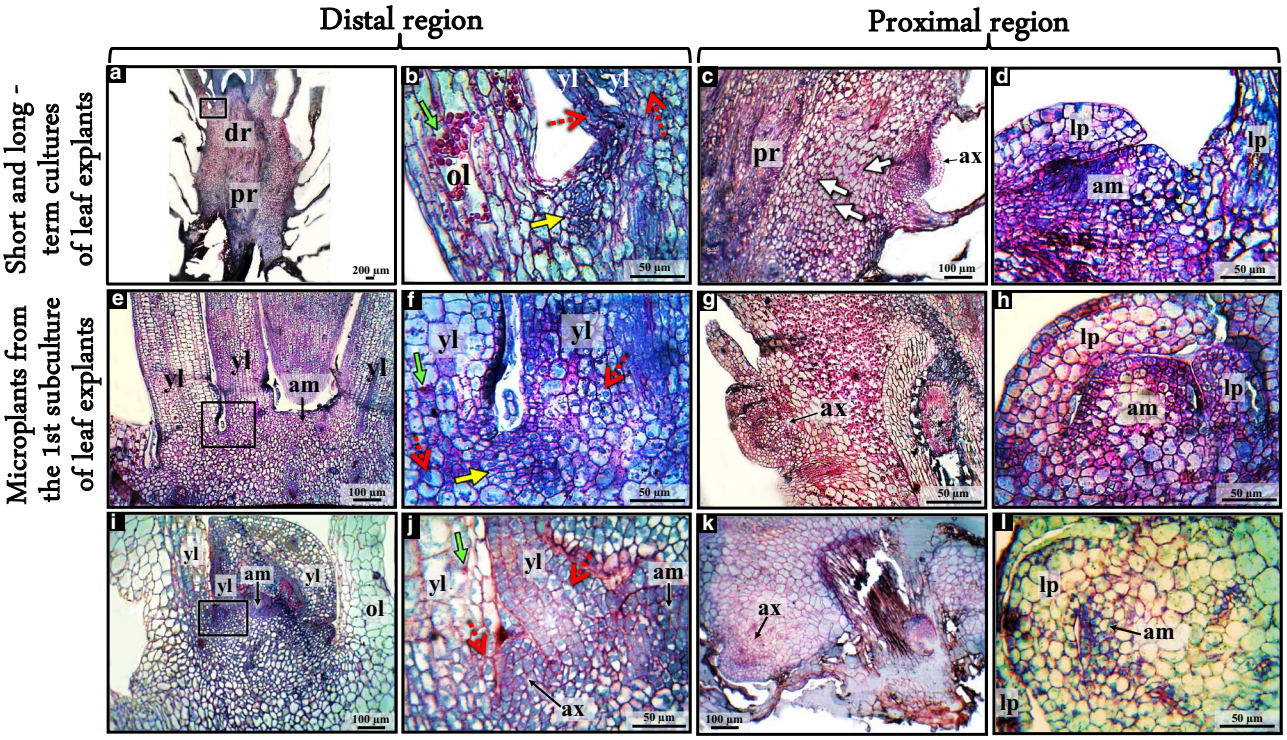

Fig. 4 Longitudinal sections of distal and proximal region with axillary buds in *N. johannis* microplants from long and short-term cultures (***a–d***) and after successive subcultures of leaf explants (***e–l***). **a** Overview of microplant. The black square represents the region analyzed in ***b***. **b** Initial development of axillary buds and amyloplast high presence and meristematic cells in the proximal region of older leaves. **c** Proximal region of shoot base with developing axillary buds. **d** Detail of the axillary bud observed in ***c***. **e**, **f** Shoot base distal region after the first subculture of leaf explants, evidencing initial development of axillary buds. The black square is detailing the event observed in ***f***. **g** Shoot base proximal region after the first subculture of leaf explants. **h** Detailing the axillary bud observed in ***g***. **i**, **j** Shoot base distal region from the second to the fifth subculture of leaf explants, showing a primordium of axillary bud. The black square is detailing the event observed in ***j***. **k** Shoot base proximal region of microplants from the second to the fifth subculture of leaf explants with the development of axillary bud. **l** Detail of the axillary bud observed in ***k***. *am* apical meristem, *ax* axillary buds, *dr* distal region, *lp* leaf primordium, *ol* older leaves, *pr* proximal region, *yl* younger leaves, *green arrows* starch, *red dashed arrow* meristematic cells, *white arrows* polysaccharides, *yellow arrow* axillary meristem. (Color figure online)

of thin cell walls, dense cytoplasm, high nuclear–cytoplasmic relationship and the presence of few and small vacuoles. These structures were observed surrounded by mucilage (polysaccharides) associated with phenolic compounds (Verdeil et al. 2007; Almeida et al. 2012; Meneghetti 2015; Graner et al. 2018) (Fig. 8h, i) and had their unicellular origin from the process of dedifferentiation and transdifferentiation (Pang et al. 2008; Kim et al. 2010; Almeida et al. 2012, 2015) mesophyll parenchyma cells except those located next to the abaxial surface, which were observed in cell death process (Fig. 8h, i). Nearby the median third, of these older leaves the distal region, we were observed proembryos (Fig. 8j–l) which also had a unicellular origin and present the same cellular characteristics of competent cells for somatic embryogenesis developed in the leaf median third (Fig. 8h, i). The somatic embryos development with unicellular origin is characterized by asymmetric divisions of embryogenesis competent cells also thin cell walls, dense cytoplasm, high nuclear–cytoplasmic relationship and the presence of small vacuoles, which decreases in quantity and their volume increases, so cells become more differentiated and specialized (Fig. 8k) (Verdeil et al. 2007; Almeida et al. 2012; Graner et al. 2018). Another event that is commonly observed is the competent cells isolation to somatic embryogenesis and/or pro-embryos through mucilage (polysaccharides) and the reduced starch content (or absence) inside their cells (Fig. 8h, i, k), because it requires high energy for this morphogenic event, and the amyloplast accumulation could be very well observed in more advanced stages of the embryonic development (Graner et al. 2018; Verdeil et al. 2007; Almeida et al. 2012). On the other hand, high amyloplasts content were detected in the parenchyma cells of the distal region, which frequently had a degenerative process and provided an energetic resource for somatic embryos induction and development (Fig. 8l) (Joy et al. 1991; Almeida et al. 2012; Meneghetti 2015). These morphogenic events corroborated with those observed by Meneghetti (2015) for the same species, however, they were described

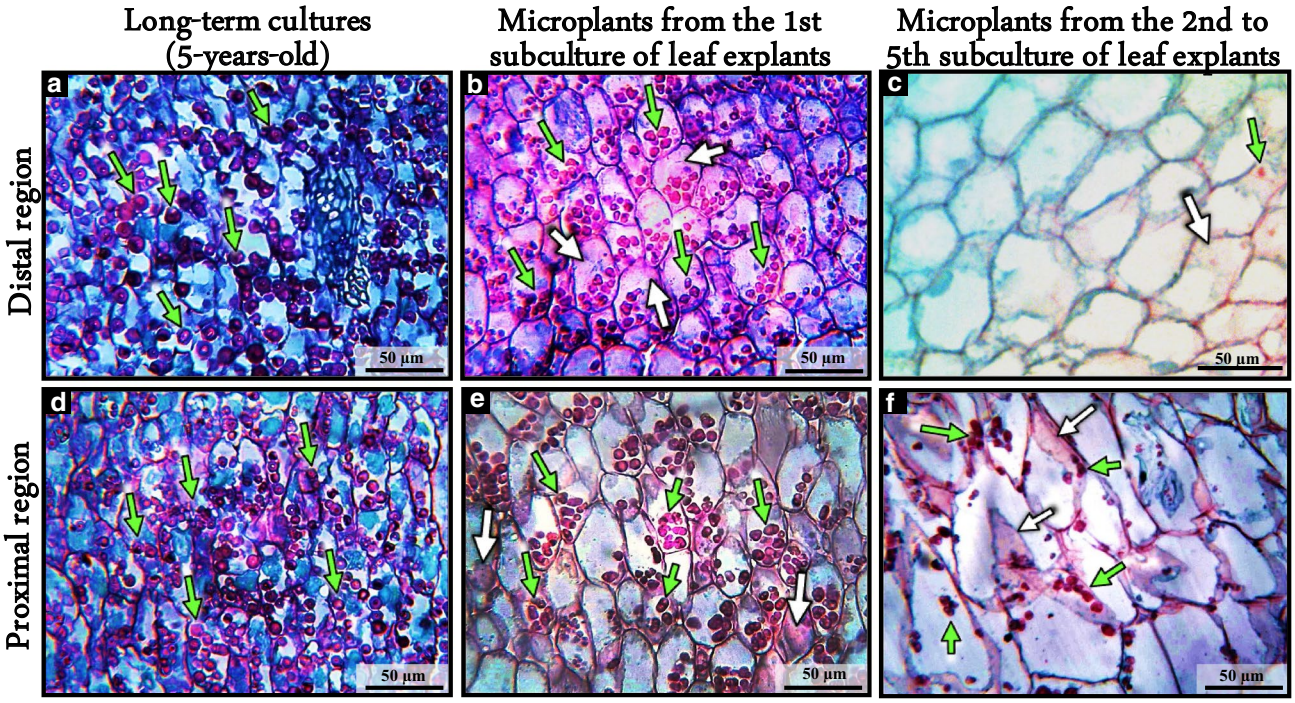

Fig. 5 Longitudinal sections of distal and proximal region in *N. johannis* microplants from long-term cultures (***a***, ***d***) and after successive subcultures of leaf explants (***b***, ***c***, ***e***, ***f***). **a**, **d** Shoot base evidencing high amyloplasts content. **b**, **e** Shoot base evidencing high amyloplasts content and reduced polysaccharides content. **c**, **f** Shoot base evidencing reduced amyloplasts and polysaccharides content. *green arrows* starch, *white arrows* polysaccharides. (Color figure online)

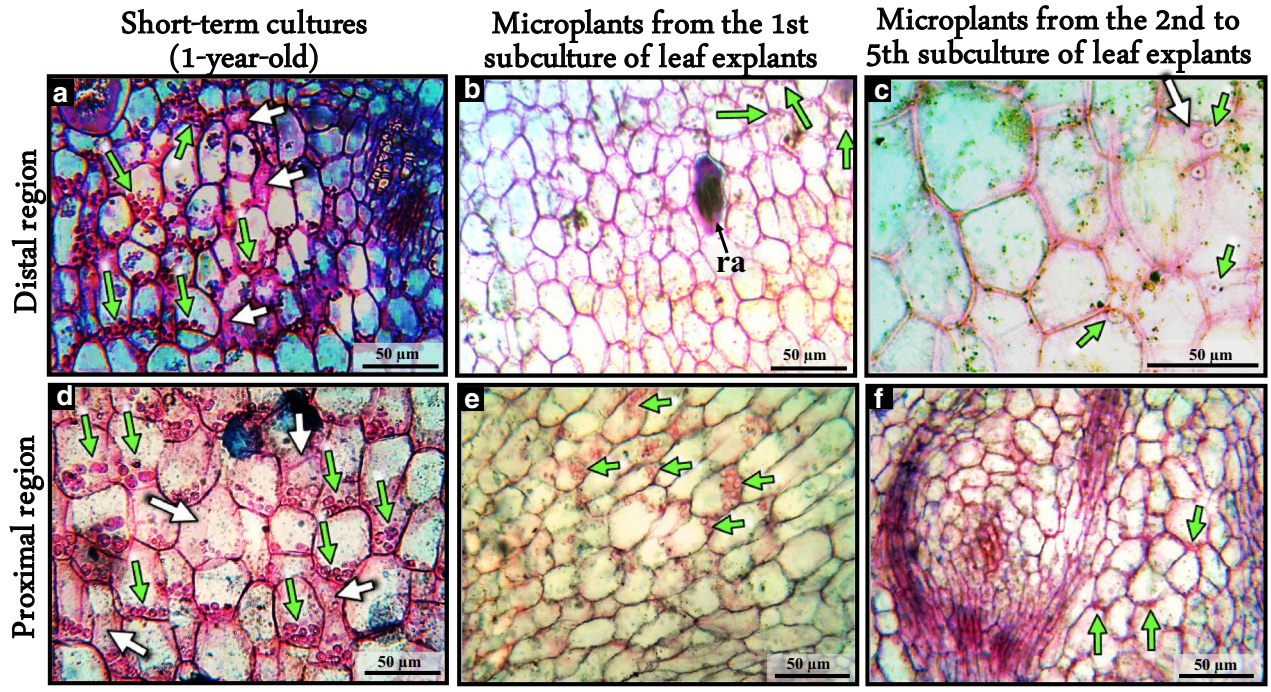

Fig. 6 Longitudinal sections of distal and proximal region in *N. johannis* microplants from short-term cultures (***a***, ***d***) and after successive subcultures of leaf explants (***b***, ***c***, ***ef***). **a**, **d** Shoot base evidencing high amyloplasts and polysaccharides content. **b**,**e** Shoot base evidencing reduced amyloplasts content and absence of polysaccharides content. **c**, **f** Shoot base evidencing reduced amyloplasts and polysaccharides content. Ra *green arrows* starch, *white arrows* polysaccharides. (Color figure online)

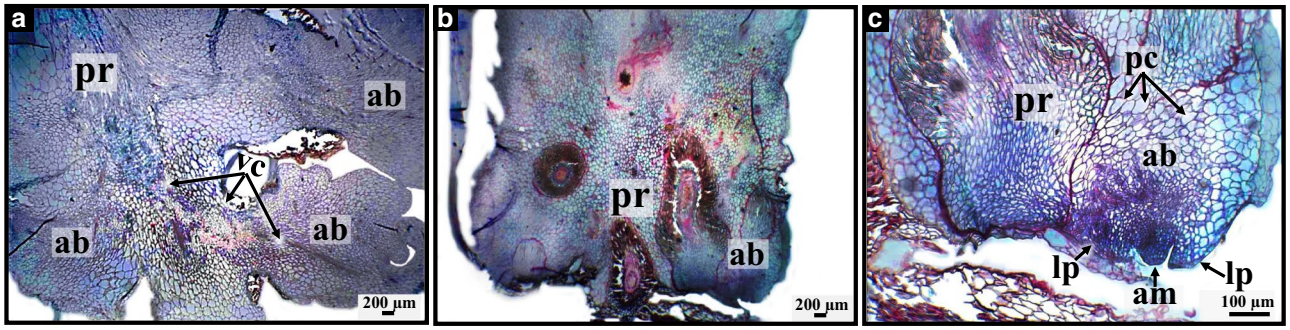

Fig. 7 Adventitious buds induced in *N. johannis* microplants from long and short-term cultures and after successive subcultures of leaf explants (***a***–***c***). **a** Direct sprouts induced from dedifferentiation and transdifferentiation of parenchyma cells at the shoot bases end proximal. **b**, **c** Adventitious buds directly induced at the proximal end of the shoot bases with the same origin as ***a***. *ab* adventitious buds, *am* apical meristem, *lp* leaf primordium, *pc* parenchymatous cells, *pr* shoot bases proximal region, *vc* vascular connection

for the first and second leaves totally expanded and were used as explants source for the multiplication, in the same morphogenic pathway as we used in this research, for the five subcultures of leaf explants (Figs. 1, 2). The viability on *N. Johanni* younger leaf explants could be corroborated by the histological and histochemical analyses observed in Fig. 4b, f, j, which show that the proximal region of the first totally expanded leaves from both the origin and independent of the subculture, we could see mesophyll parenchyma cells and epidermic cells with meristematic characteristics. These meristematic cells density is characterized by the intense blue staining detected with Naphtol blue–black due to the high cytoplasmic and nuclear proteins content and consequent intense synthesis of RNA and metabolic activity in these cell types (Almeida et al. 2012; Stein et al. 2010). The propagule induction morphogenic potential was corroborated by foliar explants ability from both origins for this (Figs. 2, 3). It is very interesting to observe that the responsive foliar explants number gradually increased, in both origins, for those coming from the short-term in vitro culture, and for the long-term in vitro culture, reaching the climax at the second and third subcultures (Figs. 2, 3). It was noted a little decrease of responsive explants on next subcultures in both origins (Figs. 2, 3). Regardless of the subculture number and the explant origin, these results corroborate with the histological and histochemical analyses, which evidenced the viability of the first and the second totally expanded leaves at older microplants as a source of explants to the multiplication and possible increase in this specie longevity. In perennial woody plants, the life span is determined by the extent of meristems persistence and their capacity maintenance for division and differentiation in new shoots and branches over the years (Munné-Bosch 2007). It is believed that many bromeliads compensates the loss of the shoot apical meristem with the axillary buds induction, however, this process seems to be not practicable, due to the high investment to protect the apical meristem of predators and particularly the leaves presence armed with thorns in terrestrial bromeliads (Benzing 2000) and saxicolous species, such as *N. johannis* (Cogliatti-Carvalho et al. 1998). In this research, the *N. johannis* microplants were cultivated in a protected environment of predators, with controlled light and temperature, but the stress due to the monthly in vitro subcultures to the renew the culture medium, and the probable aging in microplants due to the long-term in vitro culture, that could have led this specie to have a high phenotypic plasticity after the leaf explants were used for this specie regeneration. The long-term in vivo culture (field) or in vitro culture conditions (micropropagation laboratory) can lead the plant to the revigoration (reducing the physiological age) and to rejuvenation (reducing the ontogenetic age), however, the technique success is directly related to the juvenile explants use, such as the leaf explants used in this research, which were ontogenetically young (Wendling et al. 2014b). Therefore, we have to consider that a possible process for the older microplants rejuvenation happened because of the stem cells combination with the modulating ability in plants could facilitate plasticity and lead to a greater longevity resulting from rejuvenated tissues (Borges 2009). However, this requires to be evaluated with the molecular and telomere-relevant analyses related to enzyme telomerase activity (Riha et al. 2001; Watson and Riha 2011) to observe the real rejuvenation in clones because of a temporary reinvigoration, which is evaluated by morphophysiological analyses (Wendling et al. 2014b).

In this study, the *N. Johannis* microplants phenotypic plasticity was evidenced after the foliar explants of the second subcultivation on older microplants, which have seem compensated for a possible viability loss of the stem apical meristem and, therefore, microplants longer longevity, by the proximal region cells induction in older leaves began to act as niches of stem cells to pluripotency and totipotency

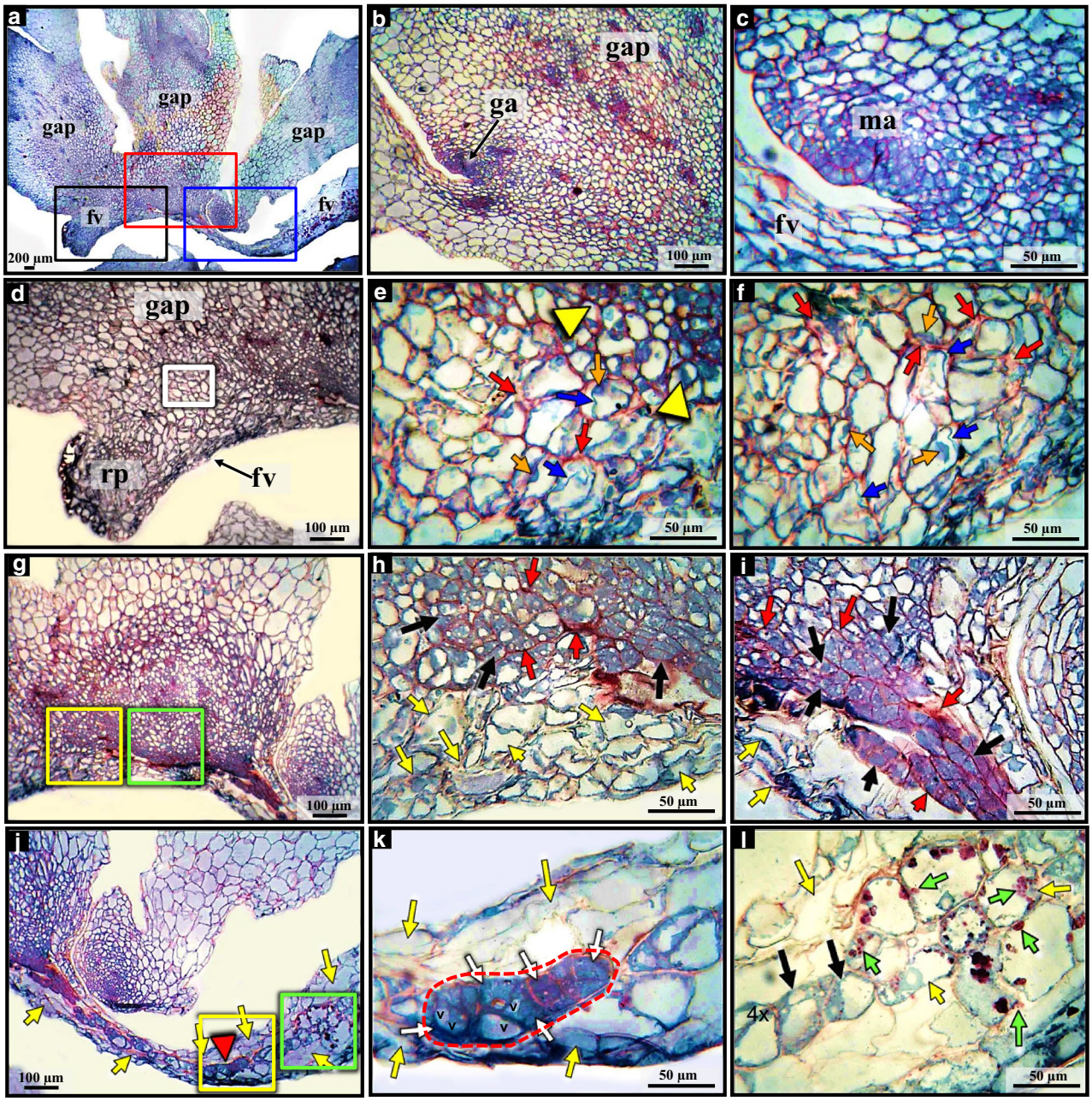

Fig. 8 Second to fifth subculture of leaf explants: longitudinal section of older leaf (***a***–***l***) separated from the basal proximal region of microplant maintained in vitro for 5 years. **a** Older leaf overview with primary adventitious buds' induction and development in advanced stage of development. The black, red and blue squares correspond to ***d***, ***f***, **e**, ***j*** photomicrographs. **b** Primary adventitious bud in early stage of development with an axillary bud. **c** Detail of the axillary bud observed in ***b***. **d** Older leaf proximal region showing the primary adventitious buds origin. The white square represents the event observed at ***e***. **e** Meristematic cells evidenced as responsible for the origin of protuberance and propagules induction. Observe cells in the cell death process and consequent mobilization of energy resources. **f** Proximal leaf extremity region and evidencing the cells in cell death process. **g** Leaf median region overview of the older leaf. The yellow and green squares correspond to ***h***, ***h*** photomicrographs. **h**, **i** Showing the competent cells presence to somatic embryogenesis, isolated by polysaccharides and polyphenols. Observe the presence of intense cell death process associated with this event. **j** Distal region overview of the older leaf. The yellow and green squares correspond to ***k*** and ***l*** photomicrographs. **k** Pro-embryo and cells in cell death process. **l** Energy resources mobilized for pro-embryos development in the distal foliar region. *am* apical meristem, *ax* axillary bud, *ol* older leaves, *pab* primary adventitious bud, *pr* proximal region, *v* vacuole, *black arrows* competent cells in the embryogenesis pathway, *green arrows* starch, *orange arrows* malformed nucleus, *red arrows* polysaccharides and polyphenols, *red arrowheads* and *red dashed lines* pro-embryos, *white arrows* proteins, *yellow arrows* cell death process, *yellow arrowheads* meristematic cells. (Color figure online)

for organogenesis and somatic embryogenesis without callus induction (Fig. 8a–l).

Depending on the stress level, some cells, tissues or organs can be conducted to the programmed cell death and to senescence process (Graner et al. 2015) or raising the cellular metabolism, leading cells to adaptation mechanisms to this physiological condition (Lichtenthaler 1998). According to Roitsch (1999), source/sink regulation (metabolism) occurs by switching on genes of sink- specific enzymes in parallel with stress defense genes. It should be note that the tissue culture technique and the collection procedure of explants can act as a stress cause (Konan et al. 2010; Graner et al. 2015), corroborating to our results, which the plants that passed by long-term in vitro culture, in two successive subcultures of leaf explants seems to have modified its metabolism, leading them to a new morphogenic potential acquisition to propagules induction. It should be note that the two subcultures of leaf explants were seemingly enough to alter the microplants metabolism and its morphogenic potential, because these remained unchanged until the experiment end, stressing that the induction and origin of the adventitious roots remained unchanged throughout the experiment (Figs. 4i–l, 5c, f, 6c, f, 7a–c, 8a–l, 9a–f, 10a–f).

The adventitious roots direct induction was observed in all experiments, those microplants that were established and maintained in vitro for 5 years, as well as in the younger (with 1 year in vitro) and successive subcultures microplants of leaf explants from both origins (Fig. 9a–f). Histological and histochemical analysis showed that all adventitious roots have been originated directly from the procambial cells meristematic activity, through many directions of asymmetric cell divisions, which are periclinal, anticlinal and oblique (Fig. 9b, c, e, f). In addition to the cell competence, we also observed in microplants older leaves the organogenesis (adventitious buds) and somatic embryogenesis (somatic embryos), other phenotypic plasticity evidence in older microplants is the response to a probable stress and/or aging as potential for somatic embryos induction in adventitious roots (Fig. 10a–f). As evidenced in the older leaves in stem bases proximal region (Fig. 8a–l), from the second subculture of leaf explants, older microplants roots also acquired competence to somatic embryogenesis from callus structures (Fig. 10a–f). The results to the propagules induction (adventitious buds and somatic embryos) in older leaves, as well as in microplants adventitious roots (somatic embryos) from the second subculture of leaf explants, remained constant until the experiment end (the fifth subculture of leaf explants) (Figs. 8, 10).

The histological and histochemical analyses evidenciced that somatic embryos had been originated from meristematic cells with embryogenic competence (unicellular origin) developed in the callus structure periphery, that was

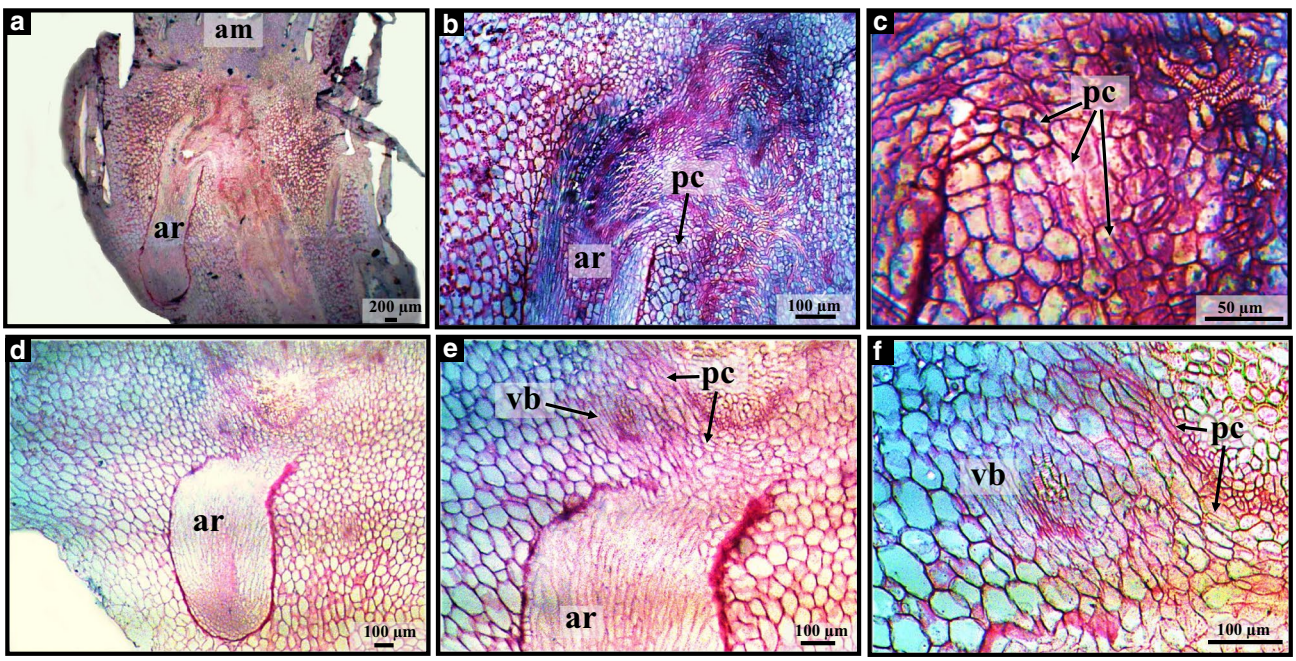

Fig. 9 **A**dventitious roots origin of both *N. johannis* microplants from long and short-term cultures (***a–c***) (***d–f***) and also in microplants from the five successive subcultures of leaf explants. **a** Older microplant overview with adventitious root development. **b** Demonstrating adventitious root procambial origin. **c** Detailing the intense meristematic activity (cell division) of procambium. **d** Newer microplants overview with adventitious root development. **e**. Demonstrating adventitious root procambial origin. **f** Demonstrating adventitious root procambial origin. *am* apical meristem, *ar* adventitious roots, *pc* procambium, *vb* vascular bundles

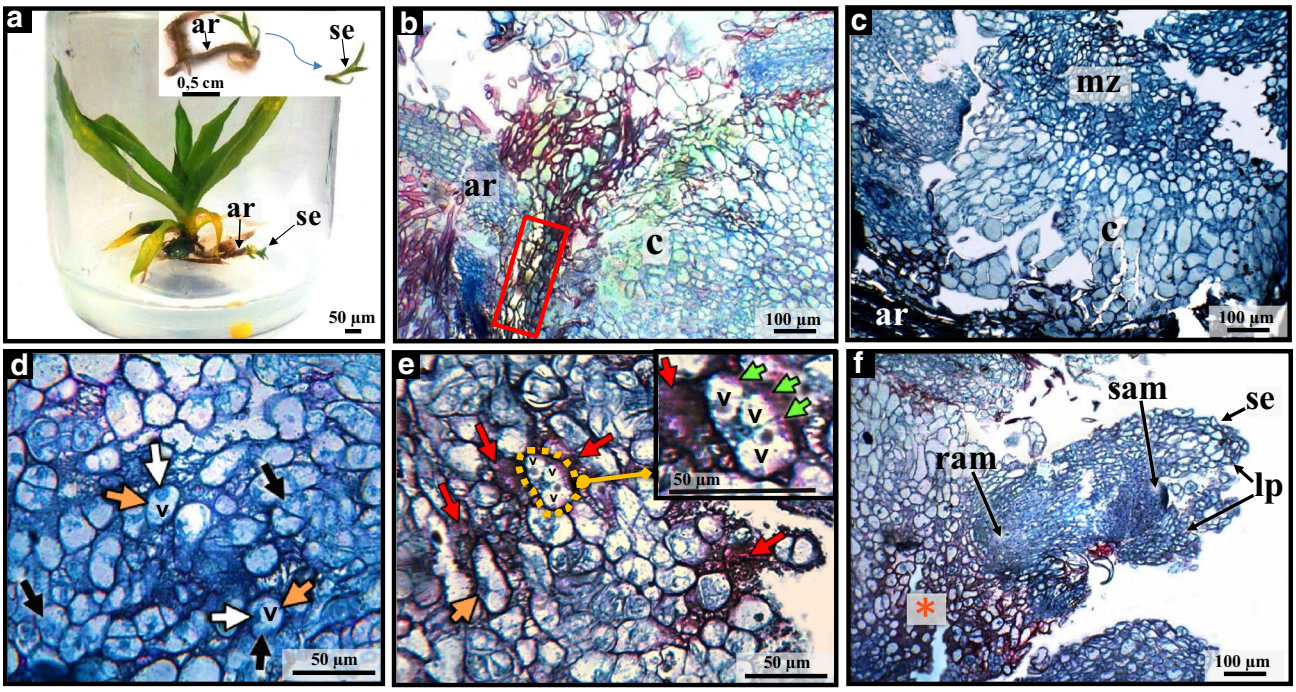

Fig. 10 Microplants from the second to the fifth subculture of leaf explants from long-term culture in vitro: adventitious root longitudinal section with somatic embryos induction (***a–f***). **a** Microplant evidencing a somatic embryo presence at adventitious root distal extremity. The figure in the upper right corner details a somatic embryo that detached itself from the root. **b** Adventitious root longitudinal section with intense epidermis cellular division and external cortical tissue (red square), originating a calogenic tissue. **c** Callus overview with a meristematic zone with more external and constituted by cells competent to the somatic embryogenesis. **d** Cells detail with competence for somatic embryogenesis **e**. Pro-embryo and competent cells to somatic embryogenesis. Observe in the upper right corner the pro-embryo with amyloplasts and polysaccharides associated with polyphenols isolating these structures. **e** Evidence of somatic embryo in development advanced stage. *ar* adventitious roots, *c* callus, *lp* leaf primordium, *mz* meristematic zone, *ram* root apical meristem, *sam* shoot apical meristem, *se* somatic embryo, *v* vacuole, *black arrows* proteins, *green arrows* starch, *orange arrows and asterisk* competent cells in the embryogenesis pathway, *orange dashed lines* pro-embryo, *red arrows* polysaccharides and polyphenols, *white arrows* nuclei. (Color figure online)

originated from the meristematic activity of the epidermis and root cortex (Fig. 10b–f). The cells with competence for embryogenesis and pro-embryos had histological and histochemical characteristics, such as their high relation nuclear–cytoplasmic (Fig. 10d, e) (Verdeil et al. 2007; Almeida et al. 2012). The meristematic cells staining with Naphthol blue–black had high protein content, suggesting high RNA synthesis and high metabolic activity in these meristematic cells (Fig. 10e). (Stein et al. 2010; Almeida et al. 2012). The stem cells asymmetric division gave rise to a bipolar structure containing cells with distinct sizes, commonly isolated by polysaccharides and phenolic compounds (Fig. 10e) (Almeida et al. 2012). Phenols, polysaccharides, and starch are directly related to embryogenic competence acquisition (Alemanno et al. 2003; Verdeil et al. 2007; Almeida et al. 2012; Rocha et al. 2012). Studies related to the role of exogenous phenolic compounds have been shown that these are related not only to the cellular dedifferentiation process (Alemanno et al. 2003), but also to first stages of the somatic embryo differentiation (Reis et al. 2008) (Fig. 9e) Almeida et al. (2012) found that the polyphenols presence was directly related to the somatic embryos induction of unicellular and multicellular origin in adventitious buds and shoot apical in peach palm. Most likely, the polyphenols observed in this study (Fig. 10e) promoted an increase on the endogenous auxin levels due to its action as an AIA oxidase inhibitor (Wilson and van Staden 1990; Hausman 1993), due to its modulation of the endogenous levels of indoleacetic acid (AIA) (Schnablová et al. 2006; Reis et al. 2008), leading the cells located at the callus periphery to the embryogenic competence acquisition and subsequent development in pro-embryos. Similarly, amyloplasts rarely identified in cells are competent to embryogenesis and pro-embryos (Fig. 10e) evidenced that this ergastic substance is mobilized and consumed even before the somatic embryogenesis initial stages, providing energy for these morphogenic events (Almeida et al. 2012). In more advanced stages for somatic embryogenic development, starch is rarely observed, because they are widely required and mobilized as an energy resource for this physiological event. Most likely, a starch

accumulation subsequent in somatic embryos could only be observed at more development advanced stages (Almeida et al. 2012). However, the competent cells isolation to the somatic embryogenesis and/or pro-embryos by mucilage (polysaccharides) is associated with polyphenols and is commonly identified in these morphogenic events, particularly with the staining technique that we used (periodic acid-Schiff stain and naphthol blue–black) (Almeida et al. 2012; Meneghetti 2015; Graner et al. 2018). This event had already been expected, because the physical isolation supports the reprogramming of genomic and cell functions, a process that is essential to the totipotency acquisition (Verdeil et al. 2007; Almeida et al. 2015). The authors had emphasized that totipotent cells physical isolation (with competence for somatic embryogenesis) occurs with plasmodesms reduction and/or absence, which are modified by the callose deposition, isolating these cells from their neighbor cells.

Many papers support the idea that somatic cells retain them plasticity, which corresponds to the ability to dedifferentiate and alter cell fate (Grafi et al. 2011). The authors pointed out that dedifferentiation represents a single transient state and its destiny is then defined how stem cells to different cellular targets through transdifferentiation, re-entering in the cell cycle and even cell death. Finally, the authors reported that there is a lot of evidence linking dedifferentiation in response to stress, in plants and animals, supporting the analyses carried out in this study.

The stress induction in plants (eg cell culture, virus infection) may lead to epigenetic and genetic variations and, consequently, to phenotypic changes as observed in the present research. Although *N. johannis* microplants were grown in a controlled environment, the stress resulting from the monthly culture medium (subculture) renewal, as well as the microplants probable aging process resulted in a considerable variability of stress over time (temporal variation) (Scheiner and Holt 2012), which could favor the cellular and phenotypic plasticity from the second subculture of leaf explants and consequent the species in vitro culture.

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Author contributions Study group in vegetable morphogenesis. EMG: formatting, conduction of experiment, histological and histochemical analyses, review, discussion of theme. ECM: formatting, discussion of theme. GFL: formatting, discussion of theme. CVA: formatting, discussion of theme. MA: supervisor, histological and histochemical analyses, review, discussion of theme.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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