Influence of exogenous putrescine on somatic embryogenesis and regeneration in litchi (Litchi chinensis Sonn.)

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Abstract

Litchi (*Litchi chinensis* Sonn.), like other ligneous plants, has long been considered a recalcitrant embryogenic species. Our previous research has shown that adding putrescine (Put) or D-Arginine (D-Arg) to the subculturing medium of embryonic callus (EC) affects EC proliferation and subsequent somatic embryogenesis (SE) in litchi. In this paper, we further confirmed that EC proliferation was significantly increased when either 0.17 mM putrescine (P3) or 2 mM D-Arginine (Ar3) was added to the control medium (M3, MS supplement with 4.52 μM 2,4-D). The subsequent induction of opalescent embryos (OEs) and opalescent dicotyledonous embryos (ODEs) was partially inhibited by Put, and the number of plantlets germinated from the OEs on the P3 medium were lower than those on the M3 medium; however, that was increased by D-Arg. Histomorphological analyses verified various developmental stages of EC proliferation in the M3, P3, and Ar3 media. On the M3 medium, an EC cell was divided into two cells first and then sequentially differentiated through multicell proembryo, globular, heart-shaped, and cotyledonary embryo stages. The EC cells on P3 and Ar3 medium were enlarged more significantly, undergoing repeated cell divisions and then forming a meristematic mass, from which OEs were initiated. The supplementation of Put into the M3 medium promoted the synthesis of endogenous Put and its conversion to Spd and Spm. The PA content in EC on the P3 medium was higher than that on the M3 medium, and the P3 medium enhanced the activity of arginine decarboxylase (ADC), ornithine decarboxylase (ODC), and diamine oxidase (DAO); however, it decreased the activity of polyamine oxidase (PAO). The PA content in the Ar3 medium was higher than that in the M3 medium. The supplementation of D-Arg to the M3 medium enhanced ADC and DAO activity but decreased ODC and PAO activity. In the other experiment, EC from the P3 medium was subsequently cultured on M3 (P3M3), P3 (P3P3), and Ar3 (P3Ar3) medium, using EC from M3 medium, and then cultured on M3 (M3M3) medium as a control. The EC proliferation rate of the P3Ar3 treatment was significantly higher than that of the other two treatments. The OEs, ODEs, and plantlets were all most elevated in the P3Ar3 treatment, followed by the P3M3 and P3P3 treatments. EC proliferation and plantlets were significantly higher than in the M3M3 treatment. When ECs were first cultured on Ar3 medium and transferred to M3 (Ar3M3), P3 (Ar3P3), and Ar3 (Ar3Ar3) media for 20 d, the EC proliferation of the Ar3Ar3 treatment was significantly higher than that of the other two treatments. Among the three treatments, EC from the Ar3Ar3 treatment showed the highest OE, ODE, and plantlet induction, followed by the Ar3M3 and P3P3 treatments. The Ar3Ar3 treatment also had the highest induction of OEs, ODEs, and plantlets compared with the other six treatments. EC from the Ar3P3 treatment had the highest Spd, Spm, and PA content, and the M3M3 treatment had the highest Put content. The Spm and PA content of EC from the M3M3 treatment was lower than in the other treatments. Among all treatments, the highest ADC, ODC, DAO, and PAO activity was found in ECs from the M3M3, P3P3, P3P3, and Ar3Ar3 treatments. These results indicate that exogenous Put or D-Arg could stimulate Put synthesis of endogenous Put by regulating the enzyme activities and then affecting the EC proliferation and SE of litchi.

Introduction

Litchi (*Litchi chinensis* Sonn.) is considered to be one of the most important fruit crops cultivated worldwide (Hu et al. 2022). Conventional breeding of litchi has several limitations because of its long juvenile period (7–8 years), low fertility, and high levels of heterozygosity common to most tropical and subtropical perennial fruit crops (Das et al. 2016). Therefore, *in vitro* techniques have potential use in litchi breeding for the large-scale cloning of elite plants. Plant regeneration *in vitro* is the basis for transgenic breeding and the rapid propagation of litchi, and its efficiency is affected mainly by the components in the culture medium. Research on media components primarily focuses on cytokinin and auxin, and very few studies have been conducted on polyamines in the in vitro regeneration of litchi. Wang et al. (2021b) found that exogenous polyamines (PAs), including putrescine (Put), spermidine (Spd), and spermine (Spm), can promote embryonic callus (EC) proliferation, maintain EC embryogenesis, and inhibit pro-embryogenesis. A high concentration of inhibitors of PA synthesis, including D-Arg and Cyclohexylamine (Cha), promoted the development of pro-embryogenesis in EC. Put showed a more significant inhibitory effect on the formation of proembryo and somatic embryos in embryogenic cell differentiation.

PAs are simple aliphatic compounds present in all living cells. They are required for growth and development in many biological processes, such as cell division, morphogenesis, secondary metabolism, chromatin organization, protein synthesis, and protein–DNA interactions (Alcázar et al. 2020, Yariuchi et al. 2021, Kielkowska et al. 2021, Chong-Pérez et al. 2012). Put is one of the main types of PA. It is a biologically active molecule widely found in eukaryotic and prokaryotic organisms. Many studies have found that exogenous Put can effectively relieve oxidative stress, reduce the damage caused by adversity stress on plant photosynthetic organs, and maintain a high level of physiological metabolism (Mandal et al. 2014, Shu et al. 2015, Vondráková et al. 2015l et al. 2018). Arginine (Arg) is an important precursor of PA synthesis in plants. Arg can be converted to Put by arginine decarboxylase (ADC) through agmatine, or by ornithine decarboxylase (ODC) through ornithine. D-Arg competitively inhibits PA synthesis through the ADC pathway (Wang et al. 2017). Subsequently, Put can be further converted to Spd and Spm by spermidine synthase or spermine synthetase. In plants diamine oxidase (DAO) and polyamine oxidase (PAO) are the key enzymes for the decomposition of Put and Spd/Spm, respectively (Aghdam et al. 2019). They not only participate in the regulation of PA homeostasis but also generate catabolic products linked to biological functions (Cona et al. 2006, Tiburcio et al. 2014, Alćazar et al. 2020). The relative activities of polyamine synthases and decomposing enzymes might affect the balance between the PA content and harmful substances, which further influence the cell membrane structure and the clearance of active oxygen, ultimately determining the direction of plant cell metabolism. D-Arg is a specific inhibitor of ADC that competes for the active centers of ADC. Our group previously found that supplementation with exogenous Put in the medium can reduce somatic embryo differentiation and plantlet regeneration. The application of exogenous D-Arg in the medium showed opposite effects, confirming that Put could regulate SE and that regulation might be related to the arginine-polyamine pathway. To further reveal the effects of Put on SE, we investigated the effects of exogenous Put and D-Arg on the contents of endogenous PAs and the activities of key enzymes responsible for PA synthesis

and decomposition in the EC of Feizixiao litchi in this study. The results further aimed to explain the response of litchi to exogenous Put under in vitro conditions from the perspective of metabolism.

Materials And Methods

Experiment 1 **Effect of exogenous Put and D-Arg on EC proliferation, SE, plantlet regeneration, histomorphological changes, polyamine contents, and associated enzyme activities**

EC proliferation

EC was induced from the anther of Feizixiao and subcultured on M3 (MS; Murashige and Skoog 1962) supplemented with 4.52 μM 2,4-D) and M4 (MS supplemented with 4.52 μM 2,4-D, 2.32 μM KT, and 29.43 μM $AgNO_3$) media as previously described by Wang et al. (2016). EC was subcultured on M3, P3 (MS medium supplemented with 0.45 μM 2,4-D and 0.17 mM Put), and Ar3 media (MS medium supplemented with 0.45 μM 2,4-D and 2 mM D-Arg) after culturing on the M2 medium (MS supplemented with 9.05 μM 2,4-D, 4.65 μM KT and 29.43 μM $AgNO_3$) for 20 d (Wang et al. 2021b). M3, P3, and Ar3 media were used for these experiments with 3 repeats for each treatment, 10 bottles per repeat, and 0.06 g EC per bottle. Put and D-Arg stock solutions were filter-sterilized by passing through 0.22 μM syringe-driven filters (HiMedia, Mumbai, India) before being added to the autoclaved cooled medium. All cultures were incubated in the dark at 25 ± 2°C. The fresh weight of the EC was calculated after 20 d, as described by Wang et al. (2021a). The EC proliferation ratio per bottle = (EC weight after 20 d of culture per bottle − EC initial weight per bottle)/EC initial weight per bottle. The EC proliferation ratio was calculated using the average of the three repeats of each treatment.

ECs were sampled on days 0, 3, 6, 9, 12, 15, 18, 21 and 24 for further analyses.

SE and plantlet regeneration

EC (20 d old) from the three media were subcultured onto T7 medium (MS medium supplemented with 0.54 μM NAA, 22.81 μM ZT, 6% (w/v) sucrose, 0.4 $g{\cdot}L^{-1}$ LH, 0.56 mM inositol, and 10% (w/v) coconut water) to induce SE. After culturing for 7 weeks, OEs with a diameter of more than 0.3 cm were counted, and ODEs were also recorded. The OEs and ODEs from the three subculture media were used to inoculate the C19 medium (MS medium supplemented with 2.85 μM IAA and 3.80 μM ABA) for maturation. After culturing for 60 d, mature OEs were transferred to the G1 medium (MS medium supplemented with 2.89 μM GA_3) and incubated under a 16 h (lightness)/8 h (darkness) photoperiod (provided by cool-white fluorescent lamps at a photon flux of 27 $\mu mol/m^2s^1$) at 25 ± 2°C for plantlet regeneration. Three replications were used for these experiments. The means of plantlet regeneration were calculated as the number of OEs forming intact plants per gram EC. The results of each treatment were expressed as the average of the three repeats.

Histomorphological analyses

For histological analysis, EC cultured on M3, P3, or Ar3 at 25 ± 2°C in the dark were sampled on days 0, 3, 6, 9, 12, 15, 18, 21, and 24. These samples were fixed for 20 h in FAA, subsequently drained stepwise with a rising concentration of ethanol, and then embedded in paraffin (Walther et al. 2022). Sections (6 μm) were stained with aluminum potassium sulfate using Ehrlich's reaction. The prepared slides were observed and photographed using a light microscope (NI/E, Nikon, Tokyo, Japan). For morphological analysis, various stages of EC were observed and photographed using a stereomicroscope (VXH-5000, KEYENCE, Osaka, Japan).

Determination of PA content

A gas chromatography–mass spectrometer (GC-MS; ThermoFisher, Waltham, MA, USA) was used to measure the content of endogenous PAs in ECs. Two milliliters of pre-cooled 10% acetonitrile solution was added to 0.2 g of the EC, which was ground and extracted for 1 h. The liquid was centrifuged at 10,000×*g* for 5 min (4°C), and the supernatant was collected as the test solution. The solution was passed through a 0.22 μM filter membrane. A 10 μL filtered solution was taken for injection, and the contents of Put, Spd, and Spm were determined. All experiments were repeated three times, with three replicates, each containing 0.2 g of EC. The values are expressed as the mean ± SD. The data were further analyzed using Data Processing System (DPS) software (version 2, Zhejiang, China) (Tang 2010).

The liquid chromatography conditions were as follows: chromatographic column: Waters ACQUITY UPLC BEH Shield RP18 (50 mm 2.1, 1.7 μM); the column with mobile phase A: 0.1% formic acid; mobile phase B: acetonitrile (containing 0.1% formic acid); isometric elution: 90% A; injection volume 2 μM; column temperature 30°C; and flow rate: 0.3 mL·min^{-1}. Mass spectrometry conditions were as follows: ion source: electrospray ionization ion source; scanning mode: full MS/AIF scanning in positive ion mode; impact energy: 35 eV; spray voltage: 3.7 kV; ion transfer tube temperature: 320°C; auxiliary gas heating temperature: 320°C; sheath gas flow rate: 40 arbs; flow rate of auxiliary gas: 15 arbs; flow rate of purge gas: 5 Arb; and auxiliary gas, sheath gas, and purge gas: high purity nitrogen.

Determination of ADC and ODC activities

The EC (0.2 g) was collected and 3 mL of pre-cooled extract buffer [50 mmol·L^{-1} phosphate buffer saline (PBS) (pH 6.3), containing 5 mmol·L^{-1} ethylene diamine tetraacetic acid (EDTA), 0.1 μmol·L^{-1} phenylmethylsulfonyl fluoride, 40 μmol·L^{-1} pyridoxal phosphate, 1% polyvinylpyrrolidone, 5 mmol·L^{-1} dithiothreitol, and 20 mmol·L^{-1} Vitamin C] was added. After grinding, the extracts were centrifuged at 12,000×*g* for 15 min (4°C). The supernatant was collected, and ammonium sulfate was added to 60% saturation. After centrifugation, the precipitate was dissolved in 10 mmol·L^{-1} PBS (pH 6.3) and dialyzed overnight at 4°C. Two volumes of pre-chilled acetone were added to the precipitate, and the precipitate was dissolved in 10 mmol·L^{-1} PBS (pH 6.3). After dialysis at 4°C overnight, the solution was centrifuged at 15,000×*g* for 15 min. The supernatant was used to determine the enzyme activity. The total volume of the reaction was 1.5 mL, including 1 mL of 100 nmol·L^{-1} Tris-HCl buffer (pH 7.5) (5 mmol·L^{-1} EDTA, 40 μmol·L^{-1} pyridoxal phosphate, and 5 mmol·L^{-1} dithiothreitol), 0.3 mL of the extracts. The system was

placed in a 37°C water bath for 2 min, and 0.2 mL of 25 mmol·L^{-1} L-Arginine or L-ornithine solution were added. After reacting at 37°C for 60 min, perchloric acid solution (PCA) was added to a final concentration of 5%, and centrifuged at 3000×*g* for 10 min. Subsequently, 0.5 mL of the supernatant was collected, and 1 mL of 2 mol·L^{-1} NaOH solution was added. After being mixed, 10 μL of benzoyl chloride was added, and vortexed for 20 s. After incubating at 25°C for 60 min, 2 mL of saturated NaCl solution was added and mixed. Then, 2 mL of ether was added and vortexed, followed by centrifuging at 1500×*g* for 5 min. One milliliter of the ether phase was collected, evaporated, and dissolved in 3 mL of redistilled methanol. The optical density was measured at a wavelength of 254 nm. All experiments were repeated three times, with three replicates, each containing 0.2 g of EC. The values were expressed as the mean ± SD. The data were further analyzed using DPS (version 2, Zhejiang, China).

Determination of DAO and PAO activities

The EC (0.2 g) was taken and added with 1 mL of 0.1 mmol·L^{-1} PBS (pH 6.5). After grinding in an ice bath, the extracts were centrifuged at 1000×*g* for 20 min. The supernatant was the enzyme extract. Then, 2 mL of 0.1 mmol·L^{-1} PBS (pH 6.5), chromogenic solution (containing 250 μL·L^{-1} N, N-dimethylaniline solution 0.2 mL and 10 g·L^{-1}, 4-aminoantipyrine solution 0.2 mL), 0.1 mL of 250 U·mL^{-1} peroxidase solution, and 0.5 mL of enzyme extract were added into a graduated test tube. Point one milliliter of 20 mmol·L^{-1} Put or Spd + Spm were added to the tube to start the reaction. After reacting at 25°C for 30 min, the optical density was measured at 550 nm. All experiments were repeated three times, with three replicates, each containing 0.2 g of EC. The values were expressed as the mean ± SD. The data were further analyzed using DPS (version 2, Zhejiang, China).

Experiment 2

Further confirmation of the effects of continuous exogenous Put and D-Arg treatment on EC proliferation, SE, plantlet regeneration, associated polyamine content, and enzyme activity

In another set of experiments, the influence of Put on EC proliferation and SE was confirmed using a complementary experiment. The treatments were set up as follows: (1) M3M3: EC cultured on M3 medium for 20 d was subsequently subcultured to M3 medium for 20 d as a control; (2) P3M3: EC cultured on P3 medium for 20 d was subsequently transferred to M3 medium; (3) P3P3: EC cultured on P3 medium for 20 d was subsequently transferred to P3 medium; (4) P3Ar3: EC cultured on P3 medium for 20 d was subsequently transferred to Ar3 medium; (5) Ar3M3: EC cultured on Ar3 medium for 20 d was subsequently transferred to M3 medium (6) Ar3P3: EC cultured on Ar3 medium for 20 d was subsequently transferred to P3 medium; and (7) Ar3Ar3: EC cultured on Ar3 medium for 20 d was subsequently transferred to Ar3 medium. There were seven treatments in total. After 20 d of culturing, the proliferation of EC was calculated, and the polyamine content and enzyme activity of EC were determined according to the methods described in *Experiment 1*. All treatments were repeated three times, with each replicate containing 0.2 g of EC. The values are expressed as the mean ± SD.

After culturing for 20 d, the ECs from the above seven treatments were subcultured onto a T7 medium. As with the previous methods, the number of OEs, ODEs, and regeneration of plantlets from each treatment were also determined. All media were adjusted to a pH of 5.8 using 1 N sodium hydroxide (NaOH) and 1 N hydrochloric acid (HCl) before autoclaving at 121°C for 20 min. All explants were incubated in the dark unless stated otherwise.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA) and are presented as the mean ± SD of all independent experiments. Using Duncan's multiple range test at the 5% probability level, the means were separated and analyzed using DPS (version 2, Zhejiang, China).

Results

Experiment 1

EC proliferation

The influence of exogenous Put on EC proliferation was assessed by transferring EC from M4 onto M3, P3, and Ar3 media. D-Arg proliferated the maximum fresh weight of EC, followed by the P3 medium and the M3 medium. EC proliferation increased more than 11.4-fold after 6 weeks of culture (Fig. 1A). From days 0–3, the EC in all treatments had no changes, and the EC gradually dried on day 6 (Fig. 2). The shape of the EC significantly began to enlarge, and the proliferation of EC on the P3 medium was more evident than on M3 and Ar3 media on day 9. The EC was yellow, with slightly coarse particles and differentiated multicellular proembryos on the M3 medium (Fig. 2). Histological observation showed that EC on the M3 medium had abundant differentiated proembryos, and the problastic cambium was obvious. Many multicellular proembryos were divided after apoptosis of the surrounding cells. The meristematic cells were small and deeply stained on the M3 medium compared with the P3 or Ar3 media (Figs. 3 and 4).

The EC was lighter yellow and grain-sized on P3 and Ar3 medium than on M3 medium. Microscopic observation showed that most of the cells on P3 and Ar3 medium were oval or elongated. The phenomenon of plasmolysis generally appears in some cells on P3 medium, while many cells on Ar3 medium contain abundant plastids. Histological observations showed that the EC cells were of uniform size and stained evenly on the P3 medium. The whole surface cells of the callus were stained thickly, divided strongly, and contained individual proembryos without problastogenesis. The EC cells were evenly stained and divided more uniformly on Ar3 medium than on P3 and M3 media.

From days 9–18, the EC moisture content on the M3 medium was gradually reduced, and the EC particles were clearly identified. The EC proliferated significantly, and the original EC became pale yellow.

Microscopic observation showed that EC cells on M3 medium had a different shape, and their sizes varied greatly. The cells were oval, spherical, elongated, or irregular in shape. The cytoplasm was rich in organelles from days 9–18.

Histological observation showed that the dividing cells of EC on M3 medium were smaller and dyed more deeply. During the later culturing period, cell division gradually weakened, and the cells began to differentiate proembryos. At the same time, the EC on P3 and Ar3 media were wetter than on M3 medium. EC on the M3 medium gradually became drier after day 6, while EC on the P3 and Ar3 media significantly became drier after days 18 and 12, respectively.

Most of the cells on the P3 medium were elongated, had abundant organelles, and were strongly divided, while most on the Ar3 medium were plump rounded cells rich in abundant cytoplasm. Histological observation showed that the cells were evenly stained on P3 and Ar3 media. The stained cells on P3 medium were slightly more profound than on Ar3 medium, where there was almost no proembryonic differentiation.

During the later cultivating process (21–24 d), the light yellow and granular EC on M3 medium became so watery that EC expanded and loosened. Macroscopic observation showed that some proembryos differentiated on the surface of EC on the M3 medium. Microscopic observations showed that the surrounding cells of the embryonic cell groups gradually degenerated and formed in reproductive isolation. The proembryos were then released.

EC on the P3 medium was more deeply yellow than that on the Ar3 medium. EC on the P3 medium became senescent and brown. Microscopic observations showed that EC cells had a degree of vacuolation and caused plasmolysis on the P3 medium. The EC cell walls were thick and lightly dyed. The cells weakly divided and did not redifferentiate the new proembryo. The original proembryos continued to differentiate into multicellular proembryos and globular, heart-shaped, and cotyledonary embryo stages. The OEs dissociated when the surrounding cells of OEs degenerated.

EC on the Ar3 medium was loose in texture, sandy on the surface, watery, and light yellow. The cell granules on the Ar3 medium were strongly divided and were darker and denser in the surface callus but lighter and looser in the center callus.

SE and plantlet regeneration

Compared to the M3 medium, supplementation with the exogenous Put or D-Arg treatments significantly increased SE (Fig. 1B). EC on the P3 medium differentiated the minimum number of OEs, with 78 per gram EC and 40 ODEs per gram EC. There were no transparent embryos, and many intensely yellow ECs on the P3 medium.

EC on the Ar3 medium differentiated the maximum number of OEs and ODEs, with 834 and 145 per gram of EC, respectively. The ODEs were about 0.1 cm, and the yellowish clumps of EC thrived on Ar3 medium.

There were 226 OEs per gram of EC and 26 ODEs on the M3 medium. The opalescent cotyledonary embryo was smaller than the globular embryo. Some transparent embryos were observed, and EC was identified by a dry and sandy appearance on the M3 medium.

Exogenous Put and D-Arg influenced plantlet regeneration (Fig. 1B). During the early regeneration stage, the fleshy cotyledon side of the proembryo appeared brilliant red, and the OEs slowly turned green. The mature OEs induced from EC cultured on Ar3 medium showed the highest plantlet regeneration, followed by M3 and P3 media. Adding D-Arg to the medium during EC subculture drastically enhanced the subsequent plant regeneration of OEs with 229 per gram EC, while adding Put into the medium caused plantlet regeneration to decline to 14. Plantlets from the M3 medium were slight, and the shoot was about 3 cm; the OEs were gradually brown. The shoot and taproot of the plantlets from the P3 medium were about 0.6 and 8 cm, respectively. The shoot and taproot of the plantlets from the Ar3 medium were about 1–5 cm, respectively.

Put content

The endogenous Put content of EC from P3 and Ar3 medium was higher than that of the control, and the Put content increased 10- and 8-fold (Fig. 5A), respectively. The content of endogenous Put on P3 and Ar3 medium sharply increased from day 0 to day 3, and then declined precipitously from day 3 to day 6. The increase rate of Put content on P3 medium following a rise–fall trend, with the highest value on day 3, and 0–9 days of incubation being significantly higher. The decrease rates on days 12–24 of incubation presented small changes.

As shown in Fig. 5A, from days 6 to 15 and days 18 to 24, the Put content in Ar3 medium slightly increased. The Put content on day 18 was lowest, and it was significantly different from that on other days. The changes in the endogenous Put content on the M3 medium showed similar trends to those on the P3 medium. The Put content followed a rise–fall–rise trend and showed significant differences at different incubation times.

Spd content

The dynamics of endogenous Spd content in EC of all treatments are shown in Fig. 5B. Spd content increased steadily on the M3 medium but fluctuated greatly on the P3 and Ar3 media. The Spd content on the P3 and Ar3 media was higher than on the M3 medium. The content of endogenous Spd on P3 and Ar3 media was 2–13 times and 1–9 times that on M3 medium, respectively. The Spd content of all treatments continued to increase from days 0 to 3, with the highest values on day 3. On P3 and M3 media, the Spd content following a rise–fall–rise–fall trend, with the highest values on days 3 and 15. From day 3 to day 12, the EC of the M3 and P3 media continued to decrease, and those of the Ar3 medium began to increase after day 9.

Spm content

The endogenous Spm content was lower than the Put content but higher than the Spm content, as shown in Fig. 5C. The Spm content of P3 and Ar3 medium was 1–17 times and 0.6–31 times that of M3

medium. From days 0 to 3, the Spm content on P3 and Ar3 media showed increasing trends but then sharply decreased after day 3. The endogenous Spm content on Ar3 medium followed a rise–fall–rise trend from days 0 to 18, with the highest values appearing on days 3 and 18. The Spm content on the P3 and Ar3 media continued to decrease after day 18. The Spm content on P3 medium had a rise–fall tendency, with the highest level on day 3. From days 9 to 24, the Spm content on the P3 medium slightly increased. The Spm content was slightly change on the M3 medium than on the P3 and Ar3 media.

PA content

As shown in Fig. 5D, changes in the endogenous PA content on the M3 medium exhibited a similar trend to Put content. The PA content on the P3 and Ar3 media was higher than that on the control; the PA content was 1–10 times and 1–9 times that of the M3 medium, respectively. The PA content on P3 and Ar3 media sharply increased and followed a rise–fall trend, with the highest value on day 3 and a sharp decline from days 3 to 6. The PA content continued to decrease after day 3. The PA content on the M3 medium showed a slightly increasing trend.

ADC and ODC activities

As shown in Fig. 6A, the ADC activities in all treatments continued to increase from day 0–3, but the rate of increase was clearly divided in two. The ADC activities in the M3 and Ar3 media increased rapidly, while those in the P3 medium increased relatively slower. On the M3 medium, the ADC activity showed a rise–fall trend, with the highest level appearing on day 3. The ADC activities on P3 and Ar3 medium showed a rise–fall–rise trend, with the highest level appearing on days 6 and 12, and 3 and 18, respectively. The ADC activity of P3 and Ar3 media was higher than that of the M3 medium. The ADC activity of the P3 medium decreased significantly after day 3, and that of the M3 and Ar3 media continued to increase after day 6.

The ODC activity of all treatments continued to increase (Fig. 6B). Among them, the ODC activity of the P3 medium increased almost linearly and had the most significant increase, with the highest level appearing on day 21. From days 18 to 24, the changes in ODC activities on the M3 medium continued to increase, with the highest level appearing on day 21, but that of the Ar3 medium decreased significantly at the same time.

DAO and PAO activities

The DAO activities of all treatments continued to increase, and the increasing rates of P3 and Ar3 media were significantly higher than those of the M3 medium (Fig. 6C). From days 0 to 6, the DAO activities of P3 medium increased rapidly, with the highest level on day 6, and then increased slightly from days 6 to 24. In contrast, the DAO activities of M3 and Ar3 media were slightly increased from days 0 to 18 and rose rapidly from days 18 to 24, with the highest level on day 24.

As shown in Fig. 6D, the PAO activity on the M3 medium was higher than on P3 and Ar3 media. The PAO activity of all treatments continued to decline slightly. The PAO activity on M3 and P3 media increased rapidly on day 18, with the highest value on day 21.

Experiment 2

EC proliferation and subsequent SE and plantlet regeneration

The proliferation of callus in P3Ar3 was highest, with a 12.18-fold increase over the M3 medium. EC on Ar3M3 and Ar3P3 media showed the lowest proliferation, and the other four treatments showed no significant difference (Fig. 7A).

When used to induce OEs, EC derived from the Ar3Ar3 medium had the highest OE and ODE induction rates and the highest plant regeneration rates (Fig. 7B–D), with 327, 55, and 87 plants per gram of callus, respectively. Compared with the Ar3 medium in *Experiment 1*, the number of OEs and ODEs and plant regeneration were reduced. The three indexes of EC on Ar3P3 medium were significantly lower than that on Ar3M3 and Ar3Ar3 media (Fig. 7B–D) and lower than or similar to M3M3 medium, indicating that the promotion of D-Arg on OE and plant regeneration was partially inhibited by Put during EC subculture. Among M3M3, P3M3, P3P3, and P3Ar3 media, the lowest OE and ODE induction rates and plant regeneration rates were found in the P3P3 medium, and the highest were found in the P3Ar3 medium (Fig. 7B–D). These results further confirmed that Put inhibits OE and plant regeneration; however, the inhibition could be partially reverted by D-Arg during EC subculture or even reverted to the level of M3M3 medium with the OE and ODE induction rates (Fig. 7B–D).

PA content

Among the seven treatments, the M3M3 medium had the highest Put content, followed by the Ar3Ar3 medium. When EC was first cultured on the P3 medium, the Put content was lower than that of the other treatments, regardless of the subsequent subculture medium (Fig. 8A). Although Spd content was the lowest among the three polyamines, it showed more variation among the treatments. There was no significant difference in Spd content among M3M3, P3Ar3, and Ar3Ar3 media; they were all lower than the other four media, in the order of Ar3P3, Ar3M3, P3M3, and P3P3 media, according to the Spd content from high to low (Fig. 8B). The M3M3 medium had the lowest Spm content, and P3P3 and Ar3P3 media had the highest. The other four treatments showed no significant difference in Spm content (Fig. 8C). The highest total polyamine content was found in the Ar3P3 medium, followed by Ar3Ar3, P3P3, and Ar3M3 media. The P3Ar3 medium had the lowest PA content; however, it was not significantly different from the P3M3 medium (Fig. 8D).

Enzyme activity

As shown in Fig. 9, EC from the M3M3 medium had the highest ODC activity, followed by the Ar3Ar3 medium. Most of the other treatments showed an insignificant difference in ODC activity. The ADC activity in EC was significantly different among most treatments, with thee P3P3 medium being the highest, followed by Ar3Ar3, Ar3P3, and P3Ar3 media being the lowest. DAO activity had the same pattern

as that of ADC. PAO activity of EC from the Ar3Ar3 medium was the highest; however, it had an insignificant difference with that of ECs from Ar3M3 and P3P3 media. ECs from M3M3 and P3M3 media had the lowest PAO activity, which was significantly lower than that of ECs from the other treatments.

Correlation coefficients of OE, ODE, plantlet, main PAs, and polyamine-related enzymes

The number of OEs, ODEs, and plantlets, the content of Put, Spd, Spm, and PAs, and the activity of ADC, ODC, DAO, and PAO were investigated using correlation analysis (Table 1). Correlation analysis showed that the correlation coefficients were 0.9375 between OEs and ODEs and 0.610 between OEs and plantlets, all reaching a very significant level. The correlation coefficients were 0.9624 between ODEs and plantlets, 0.5001 between ODEs and Put, 0.5227 between ODEs and Spm, and 0.5353 between ODEs and Pas, all reaching a very significant level. The correlation coefficients were 0.3984 between plantlets and Put, 0.5236 between plantlets and Spm, and 0.4849 between plantlets and PAs, all reaching a very significant level. The correlation coefficients were 0.5616 between Put and Spm, 0.8185 between Put and PAs, and 0.4506 between Put and ODC, all reaching a very significant level. The correlation coefficients were 0.5761 between Spd and Spm and 0.6201 between Spd and PAs, both reaching a very significant level. The correlation coefficients were 0.9278 between Spm and PAs and 0.3858 between Spm and DAO, both reaching a very significant level. The correlation coefficients were 0.6480 between ADC and DAO and 0.4868 between ADC and PAO, both reaching a very significant level. The correlation coefficient was 0.4788 between DAO and PAO, which also reached a very significant level.

Discussion

Cellular and morphogenetic events during somatic embryogenesis are controlled by a range of culture conditions and genetic effects (Kakkar et al. 2000, Paul et al. 2009). Various molecules, including PAs, have been highlighted as essential induction markers during somatic embryogenesis (Montague et al. 1978, Chée et al. 1989, Minocha et al. 2004). In the present study, exogenous Put improved the differentiation of ODE, and exogenous D-Arg increased the number of OEs and ODEs and plantlet regeneration. A possible explanation for the improved embryogenesis could be the synergistic effect between PAs and PGRs, resulting in increased stimulation of competent cells in EC. In addition, it has been reported that PAs induce high cell division and expansion, which leads to an improvement in SE (Kevers et al. 2000, Satish et al. 2015). In sugarcane, Reis et al. (2016) reported that 0.5 mM exogenous Put increased 55 somatic embryos per EC and endogenous Put level. Sathish et al. (2019) reported that 106 somatic embryos and 98 shoots per 250 mg of EC were obtained on medium supplemented with 0.23 mM Put. SE induction and maturation were increased more than two- and three-fold in the Put-supplemented medium compared to the control. Histomorphological analyses of various developmental stages verified SE from the periphery of the EC. Similar results were observed in citrus (Wu 2007), coconut (Rajesh et al. 2014a, b), tobacco (Zou et al. 2015), soybean (Arun et al. 2016), wheat (Aydin et al. 2016),

cotton (Cheng 2016), and rice (Sathish et al. 2020). These results demonstrate that exogenous Put improves SE and plantlet regeneration.

At a range of concentrations, 1–4 mM D-Arg affects EC proliferation and largely increases SE and plantlet regeneration (Wang et al. 2021b). Dai et al. (2015) reported that exogenous D-Arg significantly inhibited EC proliferation and somatic embryogenesis of L33 and L36 cotton but increased SE of C7 and Y1 cotton. In *U1mus macrocarpa* var. mongolica, exogenous Put increases the PA content and is partially reversed by the addition of D-Arg, promoting SE and regeneration (Hai 2013). The same results were obtained with other Put biosynthesis inhibitors. During DMFA or canavanine inhibitor treatment, the EC multiplicities and embryogenic potential were decreased in carrots (Mengoli et al. 1989) and *Hevea brasiliensis* (Hadrami et al. 1992). In *Lycium barbarum L.*, the EC from the Put medium was transferred to the medium supplemented with DFMA or DFMO, and regeneration was not reversed (Hu et al. 2001). Nevertheless, exogenous PAs together restore SE in carrots. These results indicate that the effect of PA biosynthesis inhibitors on SE may be species dependent.

The effect of inhibitors on Put levels contrasted with the present knowledge on Put synthesis pathways. D-Arg, an inhibitor of ADC, largely increases Put level, while diamine accumulation should have been decreased by such a treatment. The accumulation of Spd and Spm by D-Arg treatments also appears paradoxical because D-Arg should block Put synthesis, as a precursor of these two PAs. Cellular levels of PAs are known to be reduced after treatment with DMFA or other inhibitors (Minocha et al. 1999), but in our system, PAs were promoted in response to D-Arg treatment, the same as in *Araucaria angustifolia* (Steiner et al. 2007) and *anthurium andraeanum* (Sun et al. 2021). The effects of the drugs usually used on PA content are also species dependent.

The present results clarify the exact specificity of classical PA biosynthetic inhibitors. In litchi, exogenous D-Arg inhibits ODC and PAO activities one- and three-fold, respectively. PA biosynthesis inhibitors could perturb other metabolic pathways. Other pathways can also lead to PA synthesis, and the inhibition of one pathway can stimulate another to compensate for the reduced level of PA. Furthermore, treatment with PA synthesis enzyme inhibitors may increase the activity and/or the synthesis in litchi EC of other enzymes that allow PA synthesis. The turnover of these enzymes may also be reduced in response to D-Arg treatment.

Conclusion

In summary, supplementation with Put or D-Arg promoted the regeneration of litchi. Exogenous Put or D-Arg can stimulate Put synthesis of endogenous Put and its conversion to Spd and Spm, increase the activity of ADC and DAO, and moderately inhibit PAO activity. EC cultured on P3 medium was subsequently transferred to M3 and Ar3 media, and the inhibition of SE and plantlet regeneration was partially recovered. The EC cultured on the Ar3 medium was subsequently transferred to M3 and P3 media, and the promotion of SE and plantlet regeneration was partially relieved. EC continued to be cultured on P3 or Ar3 media, and the effect was partially relieved. The PA content and activities seemed

to be the same as plantlet regeneration. The supplementation of Put and/or D-Arg exhibited opposite effects, which confirmed the effects of Put.

Abbreviations

2,4-D 2,4-Dichloriphenoxyacetic acid

BA 6-Benzyladenine

CW Coconut water

D-Arg D-Arginine

EC Embryogenic callus

GA_3 Gibberellin acid

KT Kinetin

LH Lactalbumin hydrolysate

MS Murashige and Skoog

NAA Naphthalene acetic acid

NEC friable embryogenic callus

OE Opalescent embryo

ODE Opalescent dicotyledonous embryo

PAs Polyamines

PGRs Plant growth regulators

Put Putrescine

SE Somatic embryogenesis

Spd Spermidine

Spm spermine

Declarations

Author Contributions J. Wang conceived and designed the experiments. G. Wang and Y. Liu. performed the experiments. G. Wang Analyzed the data and wrote the paper. H. Li, S. Wang and F. Li contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

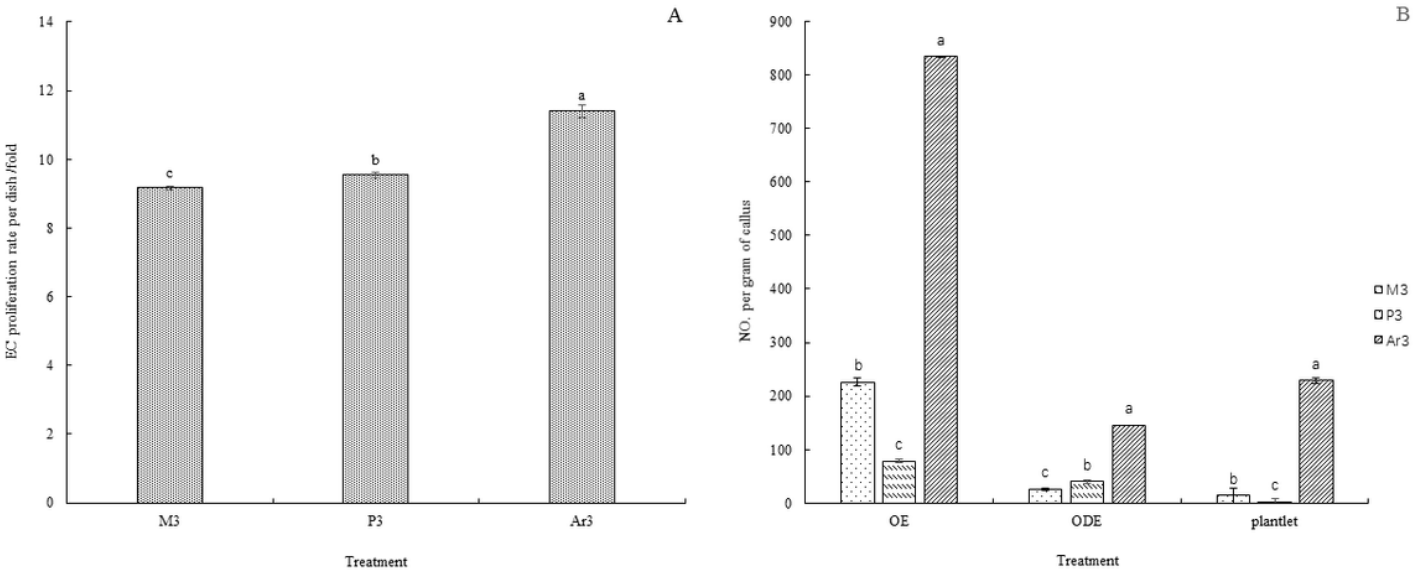

Figure 1

Effect of 0.45 mM 2,4-D in combination of 0.17 mM Put (P3) or 2 mM D-Arg(Ar3) on EC proliferation, OE, ODE and plantlet regeneration

A: After 20 d, EC proliferation rate on M3, P3 and Ar3 media was calculated as follw: EC proliferation rate = (EC weight after 20 d of culture/bottle − EC initial weight/bottle)/ EC initial weight/bottle; B: Mean number of OE and ODE formed per gram callus was calcutated after 60 d of incubation in the dark, respectively; Mean number of of plantlet regeneration was calculated after 8 weeks of incubation in the light;

Values represent the mean ± SD of three replications. Means following the same letter are not significantly different within the individual cultivars, according to Duncan's multiple range test ($p < 0.05$)

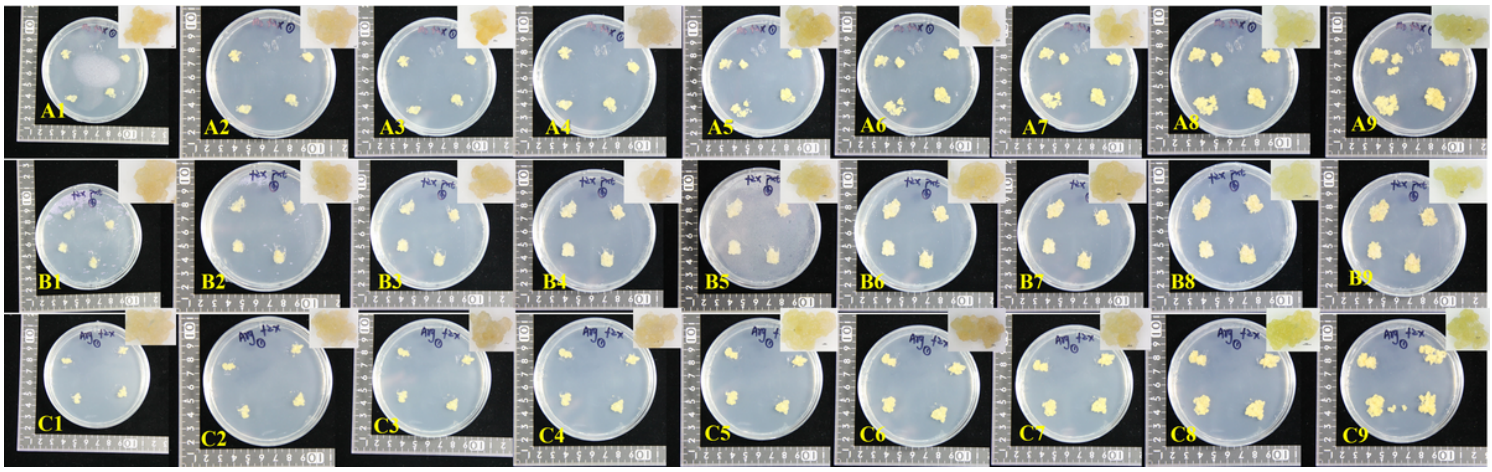

Figure 2

EC proliferation in MS medium supplemented of 0.45 mM 2,4-D (M3, as control) in combination of 0.17 mM Put (P3) or 2 mM D-Arg

A1–A9 control without PAs stimulus, A1: EC after 0 d of culture, A2: EC after 3 d of culture, A3: EC after 6 d of culture, A4: EC after 9 d of culture, A5: EC after 12 d of culture, A6: EC after 15 d of culture, A7: EC after 18 d of culture, A8: EC after 21d of culture, A9: EC after 24 d of culture; B1–B9 EC after 0, 3, 6, 9, 12, 15, 18, 21 and 24 d of subculture on supplement with Put, respectly; C1–C9 EC after 0, 3, 6, 9, 12, 15, 18, 21 and 24 d of subculture on supplement with D-Arg, respectly; A1–A2, B1–B2 and C1–C2: no change; A3–A4, B3–B4 and C3–C4: callus desiccated and proliferated; A5–A7: the callus color was gradually deeper, less water, clear particles and the callus significantly increase; B5–B7 and C5–C7: the water content significantly reduced; A8–A9: the callus inside became brown, and some protoembryos deffierentiate; B8–B9: the callus is dark yellow, and the internal callus is obviously brown and senescent; C8–C9: the callus was sandy with more water stains

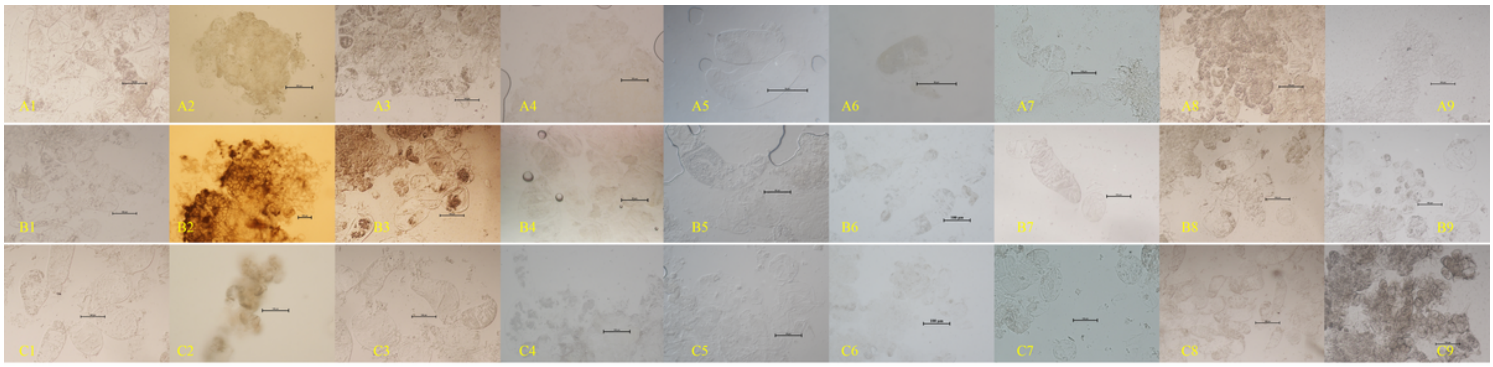

Figure 3

Morphologc characteristics of callus proliferation in M3, P3 and Ar3 media

A1–A9 control without PAs stimulus, A1: EC after 0 d of culture, A2: EC after 3 d of culture, A3: EC after 6 d of culture, A4: EC after 9 d of culture, A5: EC after 12 d of culture, A6: EC after 15 d of culture, A7: EC

after 18 d of culture, A8: EC after 21 d of culture, A9: EC after 24 d of culture; B1–B9 EC after 0, 3, 6, 9, 12, 15, 18, 21 and 24 d of subculture on supplement with Put, respectly; C1–C9 EC after 0, 3, 6, 9, 12, 15, 18, 21 and 24 d of subculture on supplement with D-Arg, respectly; A1–A4: proembryo; B1 - B4: ovoid or elongated cell of rich cytoplasm, plasmolysis; C1–C4: ovoid or elongated cell of rich cytoplasm; A5–A6: oval, spherical, elongated and irregular cells, abundant plastids, formate problastoid; B5 - B6: elongate cytoplasmic cells, divide vigorously; C5–C6: round plump cells; A7–A9: cell wall thickened; B7–B9: the proembryo continued to differentiate and grow, forming a heart-shaped embryo and beginning to dissociate; C7–C9: many proembryos

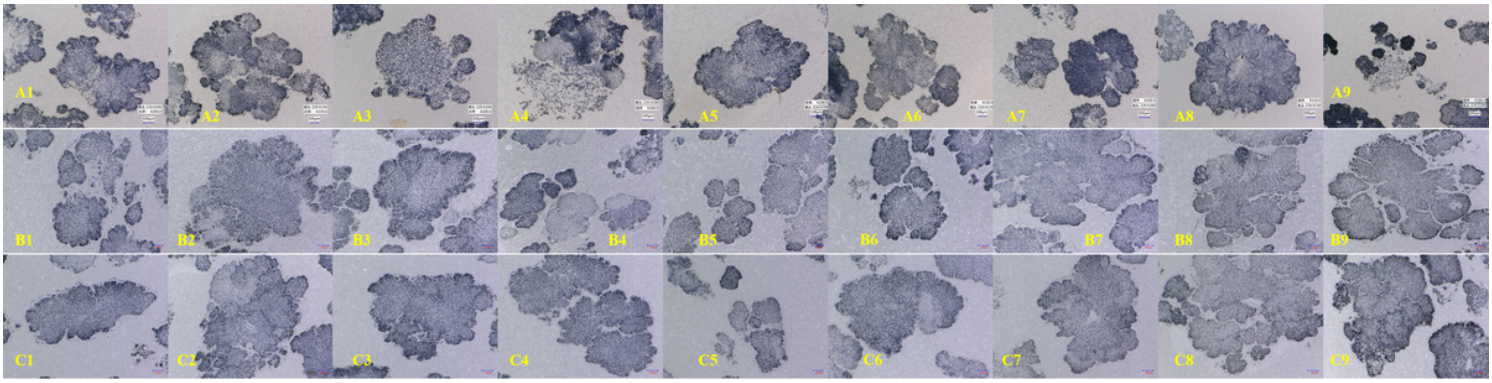

Figure 4

Histologic and morphologic reactions of callus in M3, P3 and Ar3 media

A1–A9 control without PAs stimulus, A1: EC after 0 d of culture, A2: EC after 3 d of culture, A3: EC after 6 d of culture, A4: EC after 9 d of culture, A5: EC after 12 d of culture, A6: EC after 15 d of culture, A7: EC after 18 d of culture, A8: EC after 21 d of culture, A9: EC after 24 d of culture; B1–B9 EC after 0, 3, 6, 9, 12, 15, 18, 21 and 24 d of subculture on supplement with Put, respectly; C1–C9 EC after 0, 3, 6, 9, 12, 15, 18, 21 and 24 d of subculture on supplement with D-Arg, respectly; A1–A4: proembryos; B1–B4: EC and NEC had uniform cell size and staining, the surface cells were stained the most thickly, divided quickly and contained individual proembryos; C1–C4: cells on were evenly stained and divided more uniformly, with almost no proembryo; A5–A6: the cells were smaller and more stained, cell division was gradually weakened and began to differentiate; B5–B6 and C5–C6: uniform cell staining, no proembryonic differentiation; A7–A9: proembryo gradually apoptosis, formate reproductive isolation, the proembryo began largely to dissociate; B7–B9: cells staining was slight, division was weak and no proembryo differentiation; C7–C9: the cells stain thicker and some protoembryos began to differentiate on the callus surface.

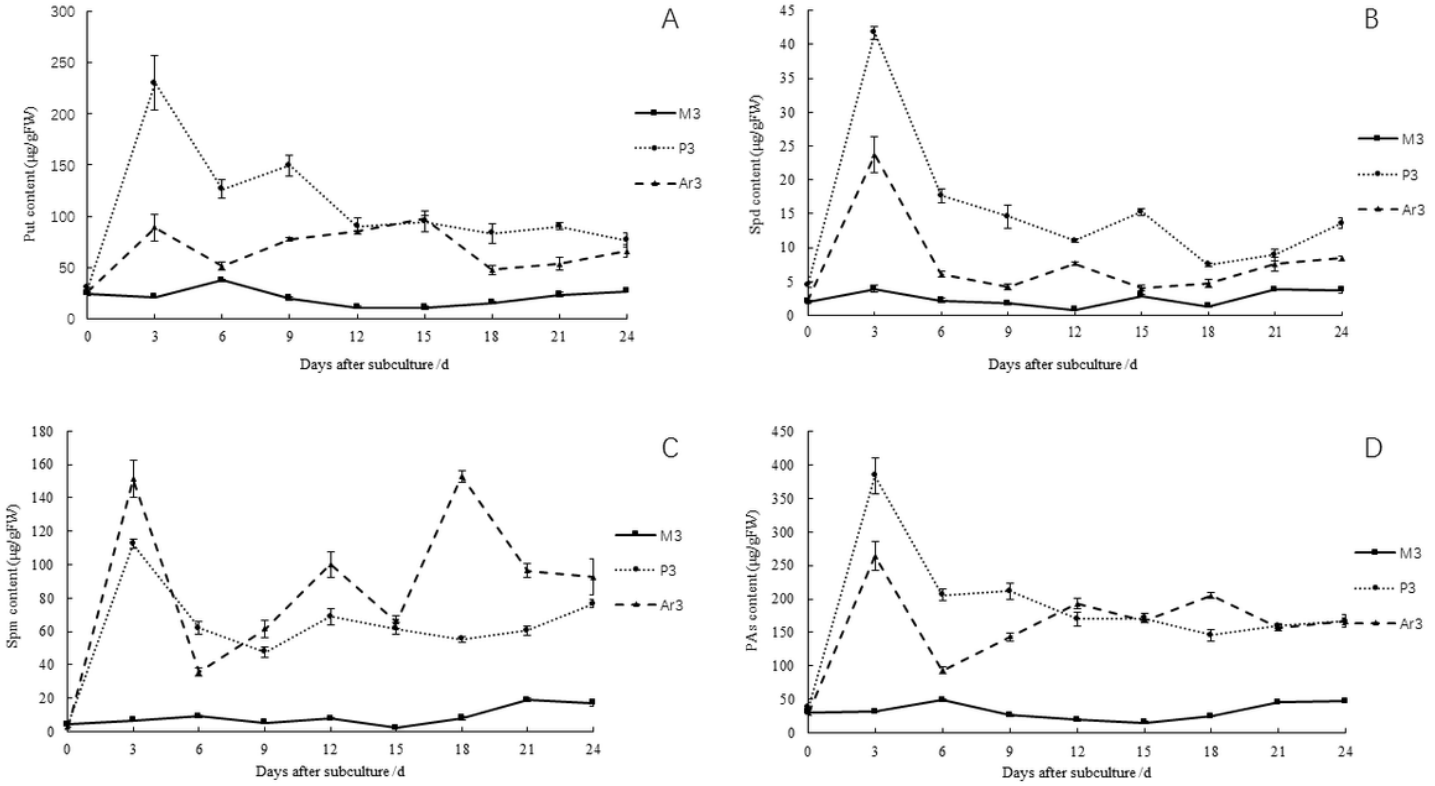

Figure 5

Effects of exogenous Put and D-Arg on the contents of endogenous Put, Spd, Spm and PAs in EC

The EC were treated with 0.45 μM 2,4-D, 0.45μM 2,4-D + 0.17 mM Put, 0.45 μM2,4-D + 2 mM D-Arg. The content of Put, Spd, Spm and PAs in EC were assayed. Values represent the mean ± SD of three replications. Means following the same letter are not significantly different within the individual cultivars, according to Duncan's multiple range test (p< 0.05). A: Put content; B: Spd content; C: Spm content; D: PAs content.

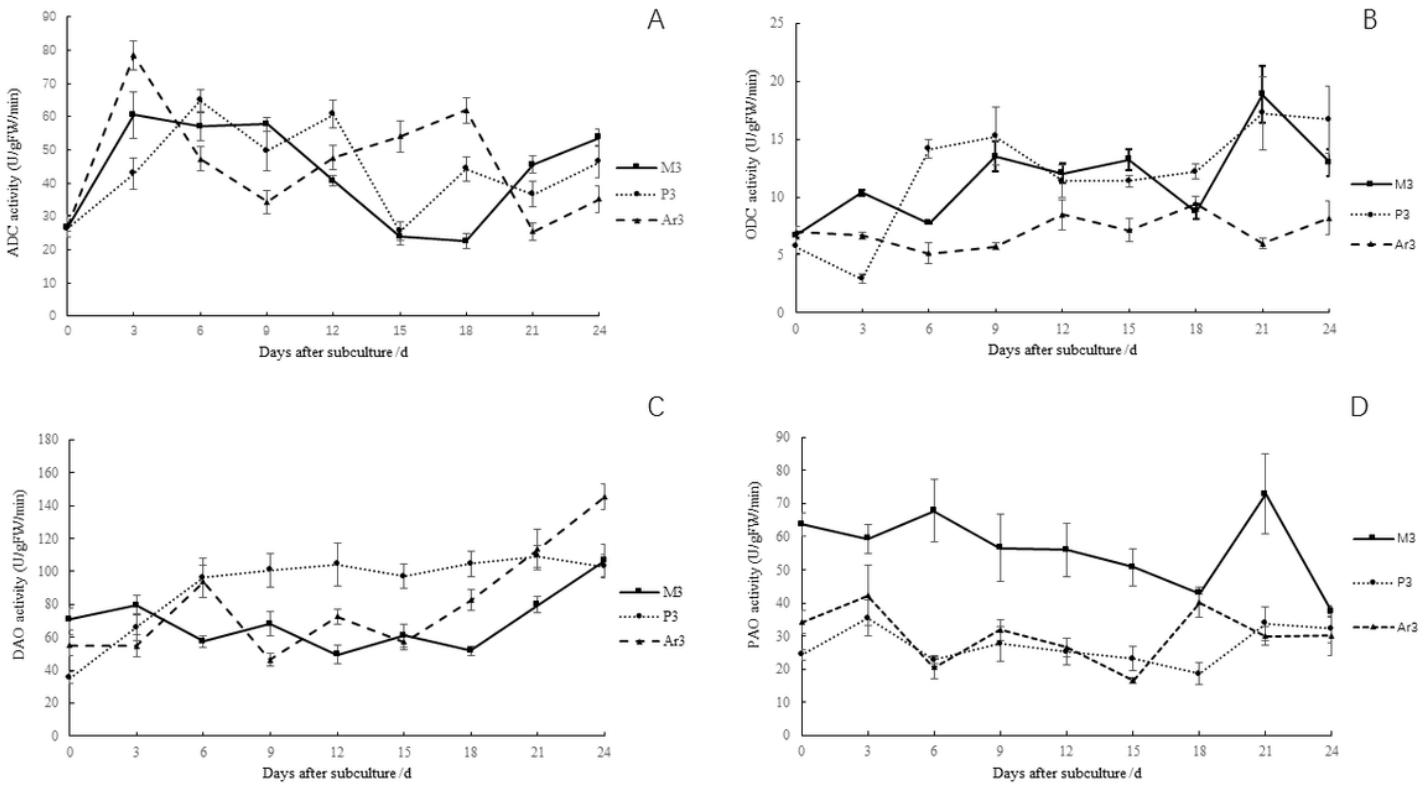

Figure 6

Effects of exogenous Put and D-Arg on the ODC and ADC, DAO and PAO activities in EC subculture

The EC were treated with 0.45 μM2,4-D (M3), 0.45 μM 2,4-D + 0.17 mM Put (P3) , 0.45 μM 2,4-D +2 mM D-Arg (Ar3). The ADC and ODC activities, DAO and PAO activities in EC were assayed. Values represent the mean ± SD of three replications. Means following the same letter are not significantly different within the individual cultivars, according to Duncan's multiple range test ($p < 0.05$). A: ADC activity; B: ODC activity; C: DAO activity; D: PAO activity

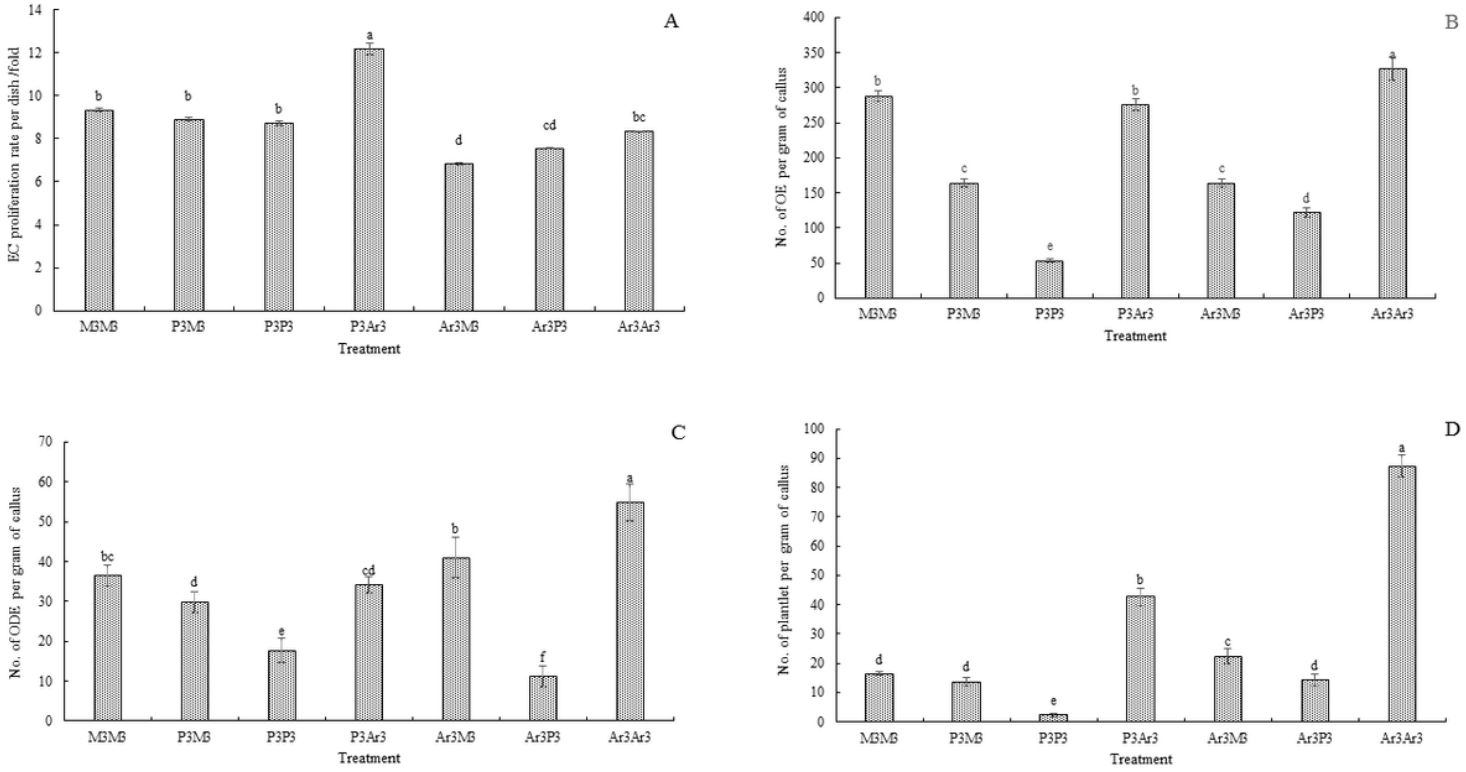

Figure 7

Effect of 0.45 mM 2,4-D in combination of 0.17 mM Put or 2 mM D-Arg on EC proliferation and OE, ODE and plantlet regeneration

A: After 20 days, the EC proliferation on M3M3, P3M3, P3P3, P3Ar3, Ar3M3, Ar3P3 and Ar3Ar3 group was calculated as follw: EC proliferation rate = (EC weight after 20 d of culture/bottle − EC initial weight/bottle)/ EC initial weight/bottle; B and C: Mean number of OE and ODE formed per gram callus was calcutated after 60 days of incubation in the dark, respectively; D: Mean number of plantlet regeneration was calculated after 8 weeks of incubation in the light; Values represent the mean ± SD of three replications. Means following the same letter are not significantly different within the individual cultivars, according to Duncan's multiple range test ($p < 0.05$)

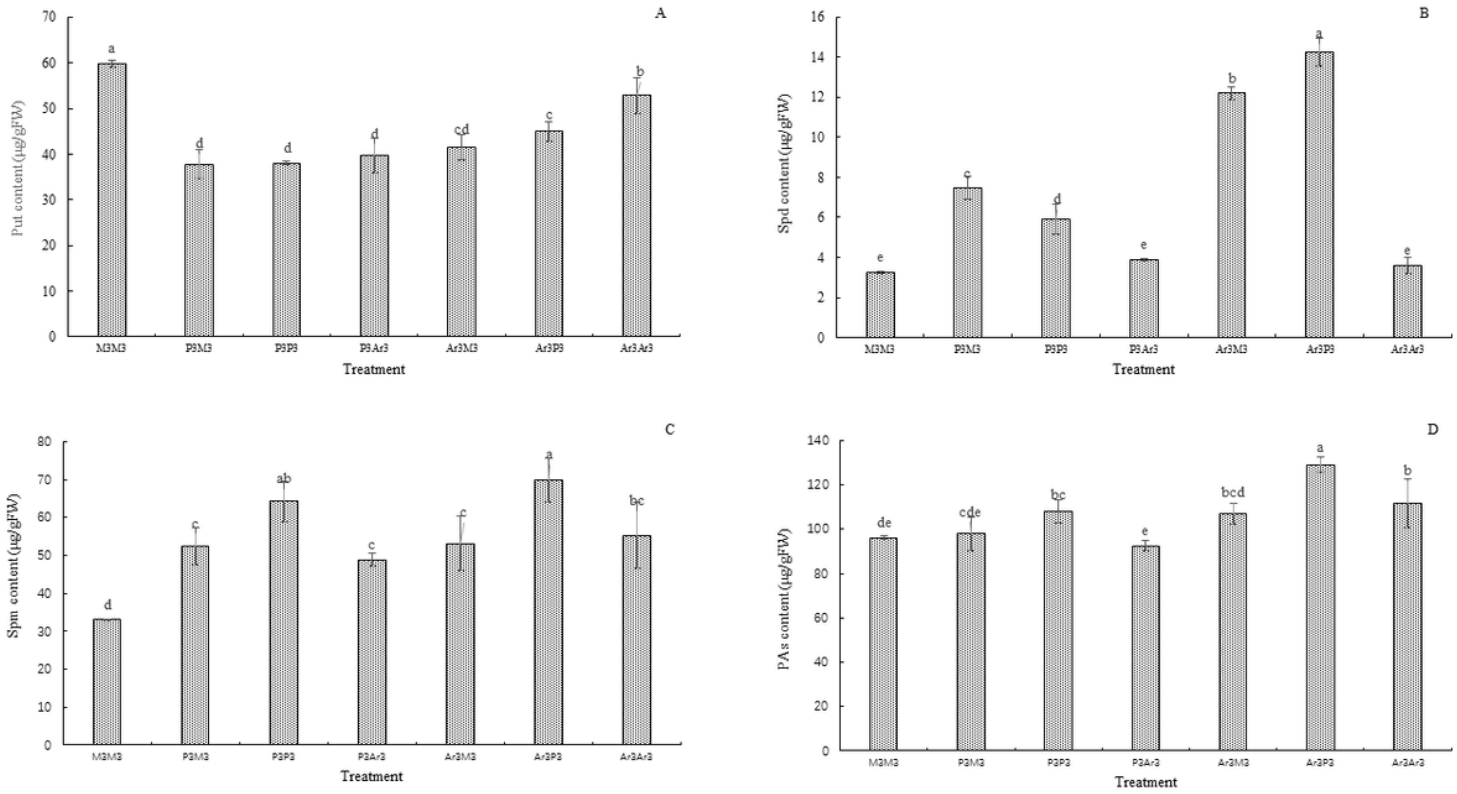

Figure 8

Effects of exogenous Put and D-Arg on the contents of endogenous Put, Spd, Spm and PAs in EC

The EC were treated with M3M3, P3M3, P3P3, P3Ar3, Ar3M3, Ar3P3 and Ar3Ar3 media. The content of Put, Spd, Spm and PAs were assayed. Values represent the mean ± SD of three replications. Means following the same letter are not significantly different within the individual cultivars, according to Duncan's multiple range test ($p < 0.05$). A: Put content; B: Spd content; C: Spm content; D: PAs content.

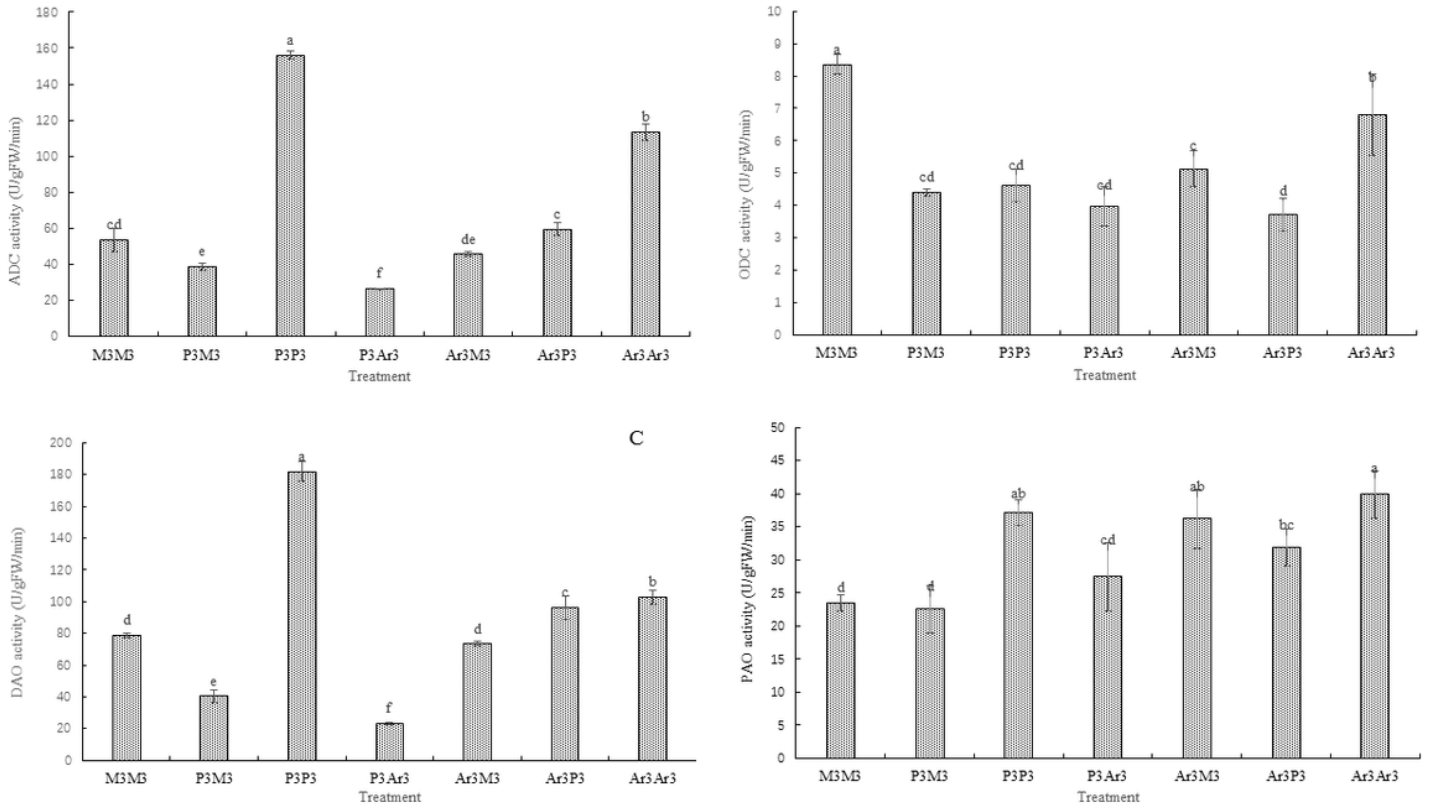

Figure 9

Effects of exogenous Put and D-Arg on the activities of ADC and ODC, DAO and PAO in EC

The EC were treated with M3M3, P3M3, P3P3, P3Ar3, Ar3M3, Ar3P3 and Ar3Ar3 media. The activities of ODC and ADC, DAO and PAO in EC were assayed. Values represent the mean ± SD of three replications. Means following the same letter are not significantly different within the individual cultivars, according to Duncan's multiple range test ($p < 0.05$). A: ODC activities; B: ADC activities; C: DAO activities; D: PAO activities.

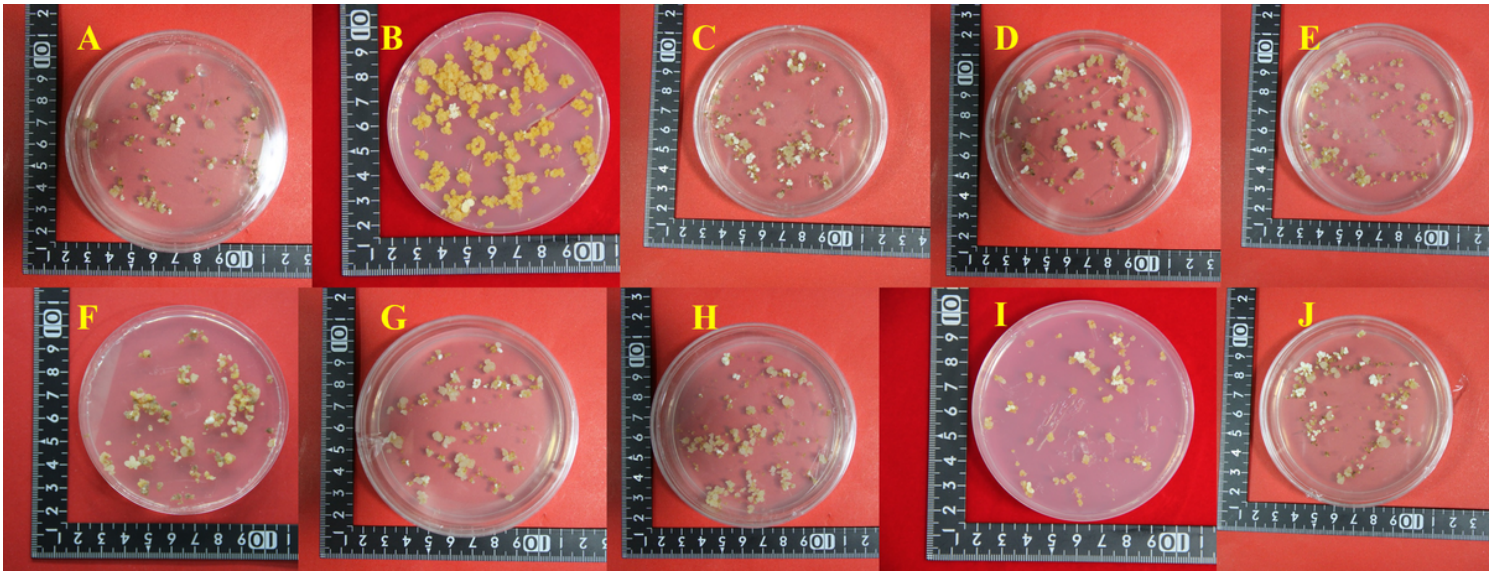

Figure 10

Effects of exogenous Put and D-Arg on SE

Initiation of primary OEs and ODEs from callus on MS medium supplemented with 0.54 μM NAA and 22.81 μM ZT, after 7 weeks of incubation in dark; A and D were control comsidered as M3 and M3M3, respectly; B: P3; C: Ar3; E: P3M3; F: P3P3; G: P3Ar3; H: Ar3M3; I: Ar3P3; J: Ar3Ar3.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.tif