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ORIGINAL PAPER

Changes in morphology and biochemical indices in browning callus derived from *Jatropha curcas* hypocotyls

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Abstract Callus browning is a typical feature of callus cultures derived from the hypocotyl of *Jatropha curcas*. Brown callus results in decreased regenerative ability, poor growth and even death. In this study, we investigated the effect of browning on callus morphology and biochemical indices. Light microscopy and scanning electron microscopy showed striking differences in callus morphology. During browning, chlorophylls and carotenoids concentrations decreased steadily. Polyphenol oxidase (PPO) and peroxidase (POD) enzymatic activities patterns were similar during callus culture with a higher activity level at week 3 compared to week 2 or later weeks. Grey relation degree analysis indicated that PPO played a more important role than POD in enzymatic callus browning. Polyacrylamide gel electrophoresis results showed differences between browning and non-browning callus. Gas chromatography–mass spectrometry results showed that saturated and unsaturated fatty acid quantities differed significantly but there was little difference in fatty acid composition between non-browning and browning callus. Differences in 17, 18.4 and 25 kDa protein concentrations were also observed in browning and non-browning callus using sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Abbreviations

LM	Light microscopy
SEM	Scanning electron microscopy
BA	Benzylaminopurine
IBA	Indole-3-butyric acid
MS	Murashige and skoog medium
PPO	Polyphenol oxidase
POD	Peroxidase
PBS	Phosphate buffer solution
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
d	Day

Introduction

Jatropha curcas L., belonging to the family Euphorbiaceae, is widely distributed in tropical and subtropical areas (Schmook and Serralta-Peraza 1997). This multi-purpose tree has a history associated with land reclaimation and for the making of feedstuff, soap, cosmetics, pesticide, anti-cancer medicine, and traditional Chinese medicine (Staubmann et al. 1999; Openshaw 2000; Lin et al. 2004; Mampane et al. 2006). Recently, attention has been drawn to the high oil content (50–60%) in *J. curcas* seeds which exceeds oil content of other biodiesel plant seeds such as cole and soybean.

In recent years, plantlet regenerations from different *J. curcas* explants have been reported (Sujatha and Mukta 1996; Sardana et al. 2000; Wei et al. 2004; Rajore and Batra 2005; Sujatha et al. 2005; Jha et al. 2007). Although reported multiplication rates were generally not low, browning during callus cultures was a major problem to

increasing multiplication rates. Tissue browning, frequently observed in callus cultures derived from mature explants of some woody plants, reduces callus growth and inhibits adventitious shoot formation (Wei and Newton 2004). The browning phenomenon is usually attributed to enzymatic browning. POD (EC 1.11.1.7) and PPO (EC 1.10.3.1) are responsible for enzymatic browning by oxidizing some phenolic substrates (Lee and Whitaker 1995). Furthermore, POD and PPO may act synergistically since PPO may promote POD activity by generating H_2O_2 from the oxidation of phenolic compound (Richard-Forget and Gauillard 1997). PPO catalyzes the oxidation of phenols to quinones that in turn polymerize to form brown pigments (Mayer and Harel 1979). POD has many roles in plant growth and development. Campa (1991) reported that POD was associated with chlorophyll degradation and lipid peroxidation in senescent plant tissues.

The aim of this study was to investigate callus browning, including changes in enzymatic activities (PPO and POD), pigment (chlorophylls and carotenoids) concentrations, differences in fatty acid composition, and cell structures differences in non-browning and browning callus derived from *J. curcas* hypocotyl explants.

Materials and methods

Plant materials, culture media, and culture conditions

Fresh seeds of *J. curcas* were collected from Panzhihua city, Sichuan Province, P. R. China. Mature seeds were de-coated and rinsed in running tap water for 30 min. The de-coated seeds were surface-sterilized with 70% (v/v) ethanol for 30 s. The seeds were then treated with 0.1% (w/v) $HgCl_2$ for 15 min, and finally rinsed five times with sterile water. The embryo axes were excised and planted on MS (Murashige and Skoog 1962) medium for germination. Fifteen-day-old seedlings were placed on MS medium supplemented with 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} BA for callus induction. Four explants were planted in each triangular flask with 40 ml callus induction medium. All media had 3% (w/v) sucrose, adjusted to pH 5.8 prior to the addition of 0.8% (w/v) agar and autoclaved at 121°C and 104 KPa for 20 min. All tissues were transferred at 2 week intervals to fresh callus induction medium. The cultures were maintained for 49 days (7 weeks) at 26 ± 2°C under the cycle of 16 h light and 8 h dark with a light intensity of 90 μmol m^{-2} s^{-1} provided by fluorescent lamps in a growth chamber.

Color changes of tissues

Callus color changes were observed visually and measured by spectrophotometer during callus culturing. Samples (0.5 g, three samples each period) were collected at the beginning of the culture period (0 day) and at 7, 14, 21, 28, 35, 42 days after culture initiation then homogenized in liquid nitrogen with 80% acetone to extract pigments. Extracts were centrifuged (12,000*g*, 20 min) and the supernatants were measured (three replicates) with a spectrophotometer at 435 nm (for chlorophylls) and 665 nm (for carotenoids) (Goodwin 1976).

Fatty acid analyses

Total fatty acids in non-browning (green) and browning (browning) callus (35 days after the initiation of culture) were extracted and transmethylated as described by Tonon et al. (2002) and Yamamoto et al. (1978). Fatty acid methyl esters (FAMEs) were dissolved in hexane for a GC-MS (Agilent Technologies, Palo Alto, CA, USA) analysis with HP-5MS column (0.25 mm × 30 m × 0.25 μm capillary) (Varian, Palo Alto, CA, USA). The flow velocity was 1.0 ml min^{-1}. The oven was programmed for 100–220°C at 5°C min^{-1}, 15°C min^{-1} to 280°C. Inlet, aux, MS (mass spectrometry) source and MS quad temperatures were 250, 280, 230 and 150°C respectively.

Preparation of samples for LM and SEM

Callus tissues, 35 days after the initiation, were cut with a freezing microtome set to 3 μm and viewed with a light microscope (Olympus BH-2). For SEM, small pieces of tissues (3–5 mm) were collected and fixed in 10:5:85 (v/v) FAA (formalin:acetic acid:94% ethanol). Fixed tissues were dehydrated in an ethanol series, infiltrated with 1:1 (v/v) Historesin:95% ethanol, substituted in pure acetone and embedded in isoamyl acetate and then dried with a critical point dryer, covered with gold, and viewed with a scanning electron microscope.

POD and PPO activity

Enzyme activities were measured in hypocotyl tissue and after 7, 14, 21, 28, 35, 42 days following callus initiation. There were three replications for each sample.

POD was extracted from 0.5 g samples as described by Li (2000). The reaction mixture contained 2.9 ml 0.05 M PBS (pH 5.5), 1.0 ml 0.05 M guaiacol, 1 ml 2% H_2O_2 (v/v), and 0.1 ml enzymatic extract. The absorbance variation at 470 nm was measured for 4 min at 20 s intervals with the value calculated per min. The results were presented as A_{470} min^{-1} g^{-1} of protein.

The PPO was extracted from 0.5 g samples as described by Zhu et al. (1990). The reaction mixtures contained 3.9 ml 0.05 M PBS (pH 5.5), 1 ml 0.1 M catechol, and 0.5 ml enzymatic extract, following the absorbance variation at

525 nm. The results were presented as A_{525} min^{-1} g^{-1} of protein.

Protein analyses

Soluble proteins were extracted from 0.5 g samples that were ground in liquid nitrogen and homogenized with a mortar and pestle in 5 ml of 50 mM Tris–HCl (pH 8.6). The homogenate was centrifuged at 12,000*g* for 15 min with the supernatant subjected to SDS–PAGE. After electrophoresis, the proteins were visualized by silver staining (Blum et al. 1987).

PPO and POD extracts were subjected by native PAGE. The concentrations of the resolving and stacking gels were 7 and 4% respectively. The electrophoresis buffer was 0.005 mol l^{-1} Tris–Gly (pH 8.3). Each sample application comprised 20 µl with 0.05% bromchlorphenol blue as the front edge indicator. Electrophoresis was performed at 4°C, after which the proteins were visualized by staining (He and Zhang 1999).

Grey relation degree analyses

A sensitivity analysis was conducted to evaluate the importance of the PPO and POD for inducing callus browning. The order of sensitivity of the PPO and POD for inducing browning was then determined based on the calculated grey correlation grades.

Changes of color during callus cultures (chlorophylls represented degree of browning) were used as reference sequence, represented as X_0, activity changes of POD and PPO during browning was taken as comparison sequence, represented as X_1, X_2, and calculated incidence coefficient and incidence degree between activity changes of PPO, POD and color changes according to (1), (2).

$$\zeta_i(k) = \frac{\min\limits_i \min\limits_k |X_0(k) - X_i(k)| + \rho \max\limits_i \max\limits_k |X_0(k) - X_i(k)|}{|X_0(k) - X_i(k)| + \rho \max\limits_i \max\limits_k |X_0(k) - X_i(k)|} \quad (1)$$

$$r_i = \frac{1}{n}\sum_{k=1}^{n} \zeta_i(k) \quad (2)$$

where ρ is the distinguishing coefficient, $\rho \in (0,1)$. Generally ρ is taken as 0.5 (Deng 1987).

Result and discussion

Color changes of tissues

The color changes were classified into the following categories: green, yellow-green, yellow-brown, brown and dark brown. In most cases the callus changed from green to yellow-green after 2 weeks of culture and thereafter changed gradually from yellow-brown to brown. The first sign of brown color appeared as small yellow-brown spots in the interior of callus at the 3rd week then gradually extended to the surface. Several brown calli were found among 4-week-old callus samples with these calli gradually increasing in number up to week 6. Gradual color variations were evident during callus browning.

The concentrations of green (chlorophylls) and yellow pigments (carotenoids) decreased step by step during callus cultures (Table 1). The green and yellow pigments disappeared at the same rate and this was manifested as a color change from green to yellow-green in callus. Color change or loss of green color was normally considered the major consequence of chlorophyll degradation (Matile et al. 1999). However, the browning discoloration is a consequence of incomplete metabolism of the chlorophyll molecule (Toivonen and Brummell 2008).

Fatty acid analyses

Jatropha curcas is an important woody oil plant, which is used extensively as source material to produce biodiesel. Monitoring changes in fatty acids during callus cultures might be an important biochemical index. Fatty acid content between non-browning and browning callus differed

Table 1 Changes in the amounts of chlorophylls, carotenoids, POD and PPO activity during callus cultures

Culture time (weeks)	Chlorophylls (A_{435})	Carotenoids (A_{665})	POD (A_{470} min^{-1} g^{-1} protein)	PPO (A_{525} min^{-1} g^{-1} protein)
0	1.37	1.52	246.2	108.6
1	0.86	1.08	224.3	98.3
2	0.65	0.83	127.6	61.9
3	0.53	0.57	186.8	86.8
4	0.42	0.41	102.3	45.1
5	0.37	0.36	87.9	41.4
6	0.31	0.33	76.2	38.7

There were three replications for pigment and protein analyses

(Fig. 1). Saturated fatty acid and unsaturated fatty acid (retention time was approximately from 10 to 16 min) had significant disparity in quantities. Concentrations of saturated fatty acid and unsaturated fatty acid in browning callus were about double than that in non-browned callus. Similar fatty acids distribution patterns were observed between non-browning and browning callus. Constancy in the pattern of fatty acid composition during cultural regime of 20 passages has been reported (Slun et al. 1971). Moreover, the proportion of saturated fatty acids was 2–4 times higher than unsaturated ones. Pandey and Gadgil (1984) reported the same conclusion in *Cucumis melo* callus using media containing coconut water. Plant growth regulators such as BA, (used in our experiments), might influence plant lipid composition and lipid metabolism in terms of fatty acids induction and alteration of compositions (Aly et al. 2008).

Alterations in cellular structure during browning

SEM revealed that non-browning and browning callus cells differ in size and appearance. Most cells are spheroid and tight in non-browning callus (Fig. 2a), whereas most of cells are loose and uneven in browning callus (Fig. 2b). Some protuberances were present on the surface of both callus types. These protuberances were large and spherical, and could be divided into two uneven parts with the smaller part on the upper portion in non-browning callus (Fig. 2c), whereas the larger part was on the upper portion in browning callus (Fig. 2d). The functions of these protuberances are unknown, but some reports suggest these protuberances sometimes contain an accessory nucleus, which is related to cell division (Häsler et al. 2003).

Frozen sections revealed non-browning callus cells arrangement was even and tight (Fig. 2e), suggesting they could develop further. In contrast brown callus cells showed disorder and a more scattered arrangement (Fig. 2f) which could affect callus development, leading to cell death.

Protein analyses

Activity analyses of both POD and PPO showed similar pattern during callus cultures (Table 1). Activities were similar in the hypocotyl and at week 1 following callus initiation, then declined at week 2 but increased at week 3 followed by a gradual decline.

The SDS–PAGE result indicated some differences between non-browning and browning callus (Fig. 3a). The concentration of 17 kDa protein was higher in the non-browning callus, but not evident in browning callus. The 25 kDa protein had a higher concentration in non-browning callus compared to browning callus, whereas the concentration of 18.4 kDa protein increased in browning callus.

The POD enzyme from plant tissues is able to oxidize a wide range of phenolic compounds, such as guaiacol, pyrogallol, chlorogenic acid, catechin, and catechol (Vamos-Vigyazo 1981; Whitaker 1994; Richard-Forget and Gauillard 1997). The POD native PAGE result (Fig. 3b) depicted that the seedlings and week 1 callus had a notable isoenzyme band (arrow indicated band in lane 0 and 1). However, there was no such band in callus from weeks 2 to 6. A possible explanation for this band in the 1st week's callus is that some hypocotyl tissue may have been present. There was no noticeable difference in enzyme activity between the

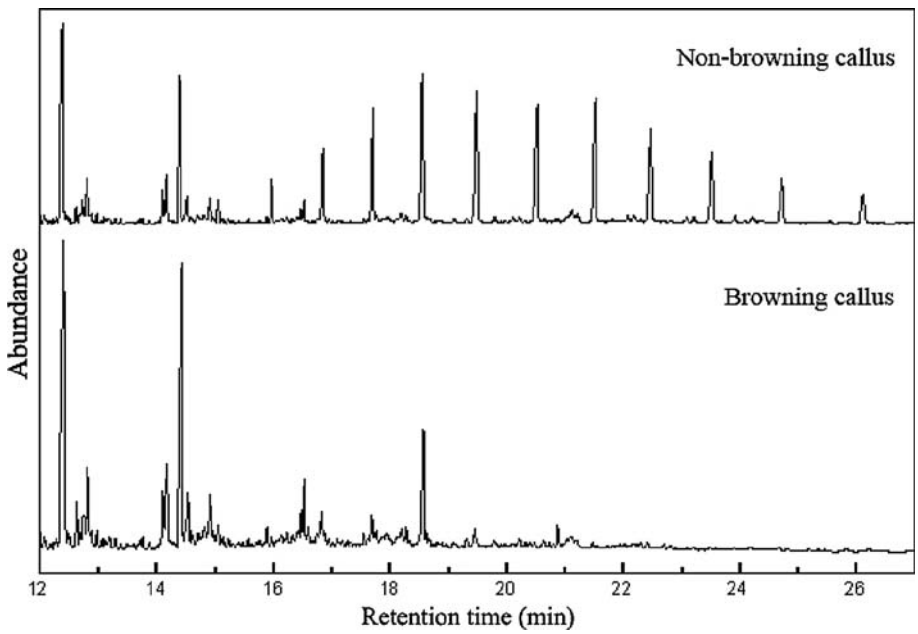

Fig. 1 Fatty acid analyses by GC–MS in non-browning and browning callus 35 days after the initiation of cultures from *J. curcas* hypocotyls

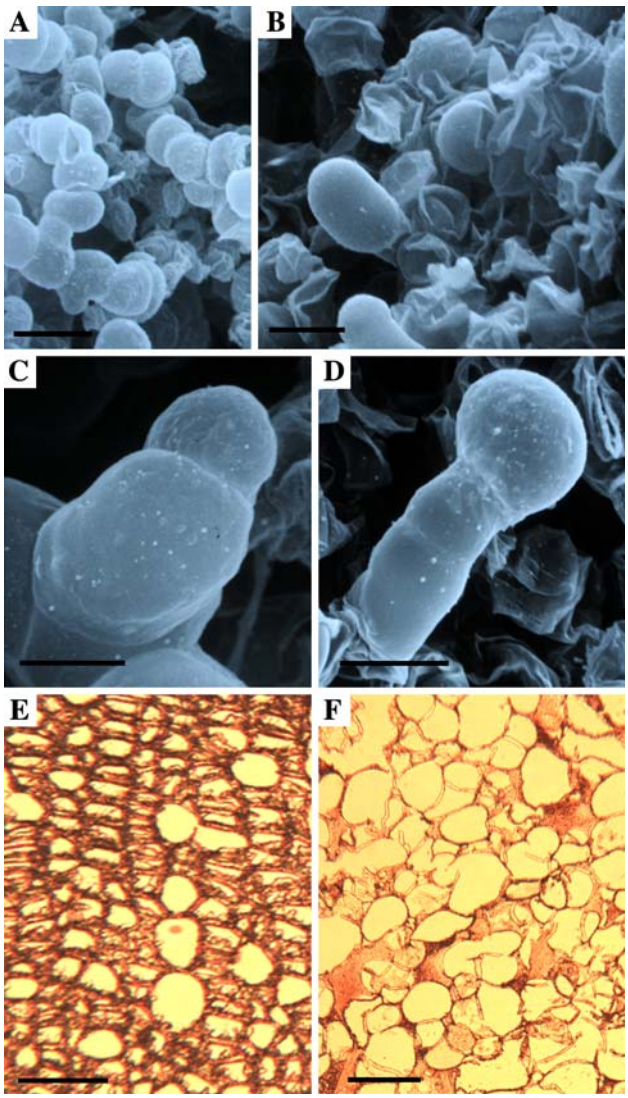

Fig. 2 Morphology of non-browning and browning callus derived from hypocotyls of *J.curcas*. Overview of **a** non-browning, and **b** browning callus. Protuberance on **c** non-browning callus with a smaller upper portion, and **d** browning callus with a large upper portion. Cell structure of **e** non-browning callus with even, tightly arranged cells, and **f** browning callus with a disordered cell arrangement. *Bars* in **a**, **b**, **d** = 50 μm; **c** = 20 μm.; **e**, **f** = 100 μm

hypocotyl and the week 1 callus. However, in following callus cultures, POD enzyme activity reached a peak in week 3 callus, then subsequently declined. Moreover there was a specific isoenzyme band (arrow indicated band in lane 3) shown in the week 3 callus. These results are similar to Kormutak's report (2001), which showed the changes in POD activity are paralleled by variations in isoenzyme composition.

The PPO enzyme catalyzes the reaction between a variety of phenolic compounds and molecular oxygen to produce ortho-diquinones. These highly reactive diquinones then react spontaneously and nonspecifically to polymerize proteins and other cellular components into the amorphous dark pigments known as melanin (Stevens and Davelaar 1996; Thygesen et al. 1994). In our study, compared to that of POD, the PPO native PAGE result (Fig. 3c) demonstrated that there were similar isoenzyme bands in seedling and the different callus cultures. Later the activity change of PPO enzyme showed a similar trend to that of the POD enzyme, suggesting they act synergistically in enzymatic browning at each callus culture period.

Grey relation degree analyses

Grey correlation analysis addresses some of the shortcomings of the traditional method (Deng 1989). The basic concept of grey correlation analysis is to determine whether a relationship among a series of data is close, based on the degree of similarity among the geometric shapes of the data series' curves with closer curves indicating greater correlation among the relative data series. The grey correlation grades are determined from the grey correlation coefficients, which measure the degree of similarity among sequences. From experiments, we can obtain sequences: $X_0 = \{1.37,0.86,0.65,0.53,0.42,0.37,0.31\}$; $X_1 = \{246.2, 224.3,127.6,186.8,102.3,87.9,76.2\}$; $X_2 = \{108.6,98.3,61.9, 86.8,45.1,41.4,38.7\}$. These sequences were calculated by formula (1) and (2), then obtained incidence degree between activity changes of POD, PPO and color changes: $r_1 = 0.62, r_2 = 0.86$. From incidence degree, we concluded that the PPO activity change was the major reason for callus browning. Browning reactions have generally been assumed to be the main consequence of PPO action on polyphenols (Martinez and Whitaker 1995), although some have attributed at least a partial role to the action of phenol POD on polyphenols (Degl'Innocenti et al. 2005). It is difficult to ascribe a significant role to POD when one of its substrates, hydrogen peroxide (H_2O_2), is generally at very low concentrations in plant cells (Veljovic-Jovanovic et al. 2002). While the mechanism of this PPO-coupled browning is not clearly understood, it is possible that the PPO-mediated generation of quinones can lead to H_2O_2 accumulation, providing a higher concentration of this free-radical species, thus enabling significant levels of POD-mediated polyphenol browning (Jiang and Miles 1993).

Conclusion

Our research is the first of its kind showing the changes in morphology and biochemical indices in browning callus derived from *J. curcas* hypocotyls. Calli color changes were observed both visually and by spectrophotometer with results showing that the chlorophylls and carotenoids in callus decreased step by step. Non-browning callus cells

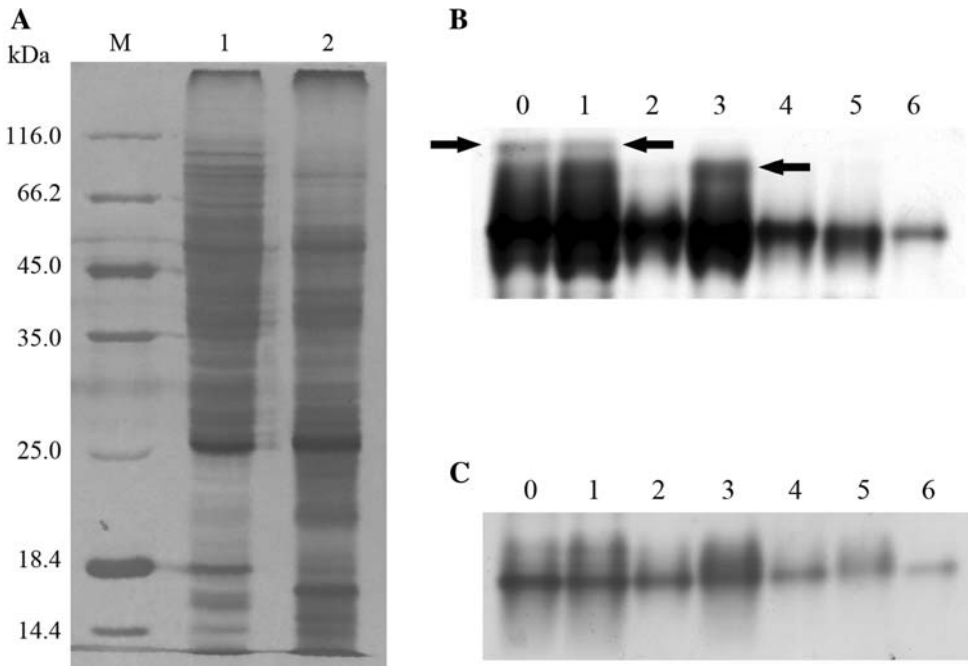

Fig. 3 SDS–PAGE and native PAGE of proteins in non-browning and browning callus derived from hypocotyls of *J. curcas*. **a** SDS–PAGE of proteins stained with silver. *Lane M*: protein marker. *Lane 1* browning callus. *Lane 2* non-browning callus. **b** Enzyme activity staining for POD after native PAGE. *Lanes 0–6* different weeks of callus cultures. **c** Enzyme activity staining for PPO after native PAGE. *Lanes 0–6* different weeks of callus cultures

had an even tight arrangement, whereas browning callus cells were disorderly and loose. Protuberances, which may be involved in cell division, differed in non-browning and browning callus. Saturated and unsaturated fatty acids differed in quantities although there was little difference in compositions in both in non-browning and browning callus. There were differences in 17, 18.4 and 25 kDa proteins between non-browning and browning callus. POD and PPO enzymes activity levels were initially high with an additional peak at week 3. Grey relation degree analysis showed that PPO played the pivotal role in inducing callus browning compared to POD.

This investigation found changes in morphology and biochemical indices in browning callus derived from hypocotyls of *J. curcas*, providing fundamental information for new research approaches to browning mechanisms, as well as methods for preventing callus browning.

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