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Physiological and biochemical changes during organogenesis and somatic embryogenesis of HBsAg-transgenic cherry tomato mutant

Cambios bioquímicos y fisiológicos durante la organogenesis y embriogénesis somática del tomate cherry mutante transgénico HBsAg

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Abstract. Leaf explants of the HBsAg-transgenic cherry tomato (Solanum lycopersicum) mutant were cultured on Murashige and Skoog (MS) basal medium, supplemented with 1.0 mg/L 6-BA and 0.05 mg/L IAA for callus induction, to clarify the physiological and biochemical characteristics of morphogenesis development. Therefore, the physiological and biochemical changes during the development of organogenic shoots and somatic embryos in the mutant were studied. Superoxide dismutase (SOD) activities of the mutant had only one peak value on the 21st day. Peroxidase (POD) activities of the mutant declined less sharply since the explants were cultured. IAA oxidase activity of the mutant increased steadily until 42 days from culturing and then decreased sharply. Malondialdehyde (MDA) of the mutant showed a significant decreasing trend after 21 days from culturing. Growth rate of the mutant was at times lower than that of the control during its callus differentiation, and the soluble protein content of the mutant callus decreased from explant cultivation until the 28th day of culture. The mutant had greater values of chlorophyll *a*, carotenoid and Chlorophyll contents than the control after 14 days of culturing, and Chlorophyll *b* content of the mutant showed a declining trend. The electrical conductivity trend of the mutant was consistent with that in the control. It indicated that in terms of the organogenesis or somatic embryogenesis pattern, protein synthesis and catabolism were very active, and a number of antioxidant enzyme activities were consistent in the early development stages of the two regeneration systems. These findings were useful for the regeneration of the mutant.

Keywords: Physiological; Biochemical; Organogenesis; Somatic embryogenesis; HBsAg-transgenic; Cherry tomato; Mutant.

Abbrevations: CAT, catalase; HBsAg, hepatitis B surface antigen; HBV, Hepatitis B virus; H_2O_2, hydrogen peroxide; MDA, Malondialdehyde; MS, Murashige-Skoog; SOD, superoxide dismutase; POD, peroxidase.

Resumen. Explantes foliares del mutante de tomate cherry (Solanum lycopersicum) transgénico-HBsAg fueron cultivados en medio basal Murashige y Skoog (MS), suplementado con 1,0 mg/L 6-BA y 0,05 mg/L ácido indol-acético (IAA) para inducción de callo, para clarificar las características fisiológicas y bioquímicas del desarrollo de la morfogénesis. Por lo tanto se estudiaron dichas características durante el desarrollo de tallos organogénicos y embriones somáticos en el mutante. La actividad de la superóxido dismutasa (SOD) del mutante solo alcanzó un valor pico luego de 21 días de cultivo. La actividad de la peroxidasa (POD) del mutante descendió menos abruptamente desde que los explantes fueron cultivados. La actividad de la IAA oxidasa del mutante se incrementó continuamente hasta los 42 días del explante, y luego se redujo abruptamente. El malonaldehido (MDA) del mutante mostró una tendencia significativa de descenso después de 21 días de cultivo. La tasa de crecimiento del mutante fue a veces menor que la del control durante la diferenciación de su callo, y el contenido de proteína soluble del callo mutante declinó desde el cultivo del explante hasta el día 28 del cultivo. El mutante tuvo mayores valores de contenidos de clorofila *a*, carotenoides y clorofila que el control después de 14 días de cultivo, y el contenido de clorofila *b* del mutante mostró una tendencia descendente. La tendencia de la conductividad eléctrica del mutante fue similar a la del control. Esto indicó que en términos del modelo de organogénesis o embriogénesis somática, la síntesis de proteínas y el catabolismo fueron muy activos, y un número de actividades enzimáticas antioxidantes fueron consistentes en los estados tempranos del desarrollo de los dos sistemas de regeneración. Estos resultados fueron útiles para la regeneración del mutante.

Palabras clave: Fisiológico; Bioquímico; Organogénesis; Embriogénesis somática; HBsAg-transgénico; Tomate cherry; Mutante.

Abreviaturas: CAT, catalasa; HBsAg, antígeno de superficie de la hepatitis B; HBV, virus de la Hepatitis B; H_2O_2, peróxido de hidrógeno; MDA, Malondialdehído; MS, Murashige-Skoog; SOD, superóxido dismutasa; POD, peroxidasa.

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INTRODUCTION

Cherry tomato (*Solanum lycopersicum*), a subvariety of common tomato, is an annual or perennial herb of the Solanaceae, tomato genus (*Lycopersicon*). Cherry tomato cultivars are usually used as materials in the research fields of genetics, physiology and molecular biology because of their delicious taste, abundant nutrient and the fact that they can be eaten raw. Recent reports of transgenic tomato have mostly focused on the variation of features such as its physiology, fruit cell structure, resistance to pathogens and nutritive qualities (Huang, 2006; Adato et al., 2009; Monteiro et al., 2011; Kausch et al., 2012).

In our previous research, an HBsAg-genetically-modified cherry tomato mutant was obtained, which had a good expression of hepatitis B surface antigen (Guan et al., 2011; Guan et al., 2012a). While developing a protocol for *in vitro* propagation of cherry tomato mutant expressing the HBsAg, we discovered that the transgenic cherry tomato mutant displayed a different morphology than non-transgenic cherry tomato. The two morphogenesis ways (shoot organogenesis and somatic embryogenesis) simultaneously appeared in mutant material (Guan et al., 2012b).

To clarify the physiological and biochemical mechanism of morphogenesis development in the transgenic cherry tomato mutant, leaf explants of the mutant were cultured *in vitro*, and the variations in the physiological and biochemical metabolism were analyzed during its callus formation and development. This paper analyzed the developmental changes of the mutant callus at the physiological and biochemical levels. This would allow to verify the differences between non-transformed cherry tomato and transgenic mutant. In turn, it helps to know more about the role of HBsAg gene, and the use of transgenic plant mutant.

MATERIALS AND METHODS

HBsAg-transgenic mutant and untransformed plant (NK). The transgenic mutant (N244) was obtained as described in Guan et al. (2012a). Since the seeds of the existing mutant were sterile, the transformed mutant was subcultured for further research. Seedlings of non-transformed cherry tomato plants were used as the control NK.

Callus culture conditions. The explants from 3-week-old leaves of transgenic mutant and the control NK were cultured on MS medium (Murashige & Skoog, 1962), added with 1.0 mg/L 6-BA and 0.05 mg/L IAA, under a 14 h photoperiod, irradiance of 2500 lx, and temperature of 25 ± 2 °C.

Determination of physiological indexes. Calli cultured on medium were harvested after 0, 7, 14, 21, 28, 35, 42 and 49 days of culture, and physiological indexes were determined. SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the protocol of Giannopolitis and Ries (1977). Hydrogen peroxide levels from the samples were determined spectrophotometrically at 415 nm according to Brennan & Frenkel (1977). CAT activity was measured by following the decomposition of H_2O_2 at 240 nm according to the method of Aebi (1974). Peroxidase (POD) activity was analyzed by the guaiacol reduction method (Zhang & Qu, 2001).

Chl *a*, Chl *b*, Chl *a* + Chl *b*, and total carotenoid (Car) contents were determined as described by Porra et al. (1989). Samples of approximately 0.2 g of calli were homogenized in 80% acetone at 4 °C. The homogenates were centrifuged, and fluorescence was measured at 662, 645 and 440 nm with a microplate spectrophotometer. Chl contents of Calli were obtained according to the formula given by Zhang & Qu (2001).

Soluble protein content was estimated using Bradford's method. Malondialdehyde (MDA) content was determined by the thiobarbituric acid method, and IAA oxidase (IAAO) was measured in percentage of IAA destroyed (100% IAA is in the control without enzyme fraction) calculated from the absorbance of Salkowski's reagent at 530 nm. Both methodologies were previously described by Zhang & Qu (2001). Relative electrical conductivity was estimated following the method of Li (2000).

Statistical analysis. The physiological indexes above in each treatment were repeated at least three times with 30~40 callus pieces. Data presented are means ± standard error for three or more independent experiments.

RESULTS

As a direct indicator, the changes of callus fresh weight can reflect the ability of cell proliferation. Both the mutant and the control grew slowly during 0-14 days of culture (Fig. 1A). Compared with the fast-increasing control, the growth rate of the mutant sharply lowered during 21-28 days of culture, and then exhibited a rapid increase of fresh weight. The reason for the variation of the mutant could be that the first organogenesis way of the mutant resulted in a reduced growth rate, and subsequent emergency of the somatic embryogenesis way increased callus fresh weight.

The mutant callus had obviously different change of soluble protein content from the control. Soluble protein of the control callus rapidly decreased at the early culture stage, increased at the embryonic callus stage, and then fell after the formation of the cotyledon embryoid (Fig. 1B). By contrast, the changing trend of the mutant was opposite to that of the control from the cultured 14th to 35th day. During other cultured periods, soluble protein content of the mutant declined.

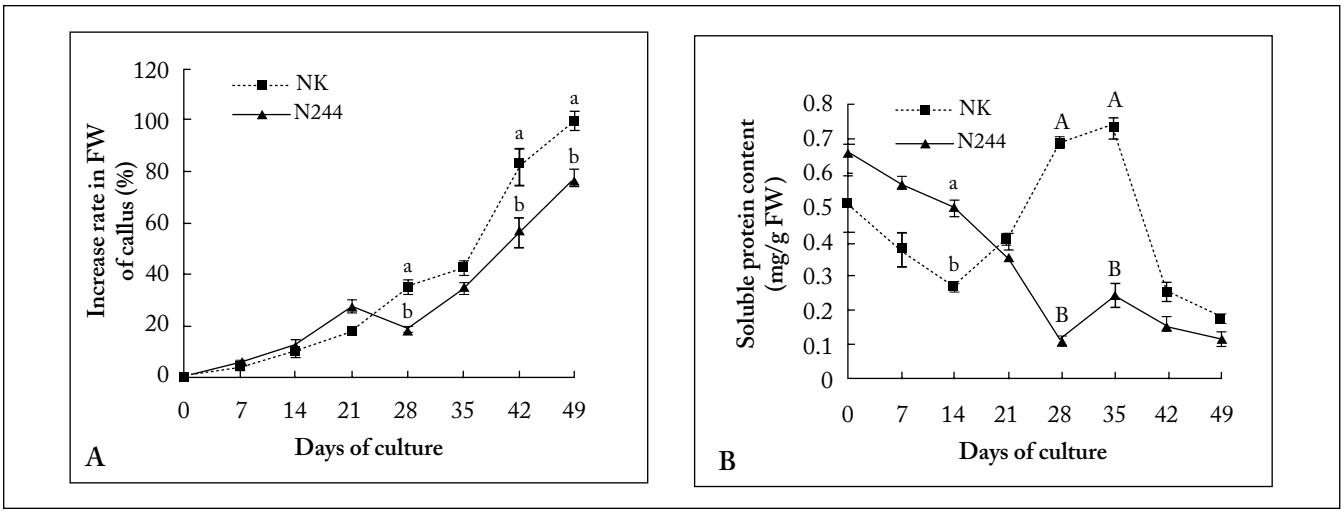

Fig. 1. Changes in levels of cell growth (A) and soluble protein (B) in the callus from both the mutant (N244) and the control (NK). FW: fresh weight. Mean values and SE were calculated from three independent experiments. Different lowercase letters indicate significantly different values at p=0.05. Different uppercase letters indicate significantly different values at p=0.01 (Tamhane's multiple range test).
Fig. 1. Cambios en los niveles de crecimiento celular (A) y proteína soluble (B) en el callo del mutante (N244) y el control (NK). FW: peso fresco. Los valores promedio y SE fueron calculados de tres experimentos independientes. Letras minúsculas diferentes indican diferencias significativas a p=0,05. Letras mayúsculas diferentes indican valores estadísticamente diferentes a p=0,01 (prueba de comparación de promedios de Tamhane).

Chlorophyll, which is the most important pigment in the process of photosynthesis, plays a very important role in the process of light absorption, transmission and transformation. Its content is closely related with the level of photosynthesis and has a direct impact on plant growth. The content of every chloroplast pigment in both the mutant and the control gradually decreased during 0-7 days of culture, and increased after 42 days (Fig. 2A-D). However, there were some significant differences in chloroplast pigment content between the mutant and the control. First of all, the mutant had the maximum value of chlorophyll *a*, carotenoid and Chlorophyll content on the cultured 14th day, while the control had the maximum value of every pigment content on the cultured 28th day (except for the carotenoid content). Secondly, Chlorophyll *b* content of the mutant showed a declining trend (Fig. 2B). In addition, during 14-42 days of culture, each pigment of the mutant also showed a reduced content quality; by contrast, the content on the control first increased and then decreased.

The balance state of reactive oxygen could be embodied by antioxidant enzyme activities (Basu et al., 2010). The antioxidant enzyme activities of the mutant had distinct differences from those in the control during the callus development period (Fig. 3A-E). Superoxide dismutase (SOD) activities of the mutant callus reached the maximum on the 21st day (Fig. 3A). Peroxidase (POD) activities of the mutant callus declined less sharply since the explants were cultured than those in the control (Fig. 3B). The overall change tendency of Catalase (CAT) activities was not obviously different when comparing the mutant and the control (Fig. 3C).

Hydrogen peroxide (H_2O_2) content of the mutant increased first and then declined with a maximum on the cultured 14th day. These changes were consistent with those in the control (Fig. 3D). MDA content of both the mutant and the control declined during the whole culture stages, and the mutant showed a significant decreasing trend during the first 0-20 days from culturing (Fig. 3E). In plant tissue culture, IAA oxidase activity can regulate the content of IAA in callus, and thus affects the growth situation of the callus. During the callus induction and differentiation stage, IAA oxidase activity of the mutant did not increase during the first 42 days of culturing, and subsequently it fell steeply (Fig. 3F).

Plant cell membrane plays an important role in maintaining cell micro-environment and normal metabolism. Changes of electrical conductivity can reflect the strength of the cell membrane permeability. The electrical conductivity trend of the mutant was consistent with that in the control before the 14th day from culturing (Fig. 4). Both of them had the maximum value on the cultured 14th day. In the subsequent culture stage, however, the change of the mutant was more significant than in the control. It is indicated that the effect of a different morphogenesis pathway on electrical conductivity was very evident.

DISCUSSION

The literature on antioxidant enzyme activities in the development of organogenesis is scarce. In this paper, the dynamic variation of antioxidant enzyme activities were systematically

studied during the occurrence and development of callus cells. We examined the main characteristics of the transgenic mutant during callus development, and changes of physiological indexes at different development stages. Results revealed physiological development mechanisms of the transgenic mutant callus.

Callus differentiation and morphogenesis is a complex process, which may result in some intracellular gene regulatory activities. As a result, changes in cell structure, function and physiological and biochemical characteristics are expected. Typically, cell physiological and biochemical changes always precede visible variations in morphology. Combined with previous experimental results (Arnaldos et al., 2002; Casanova et al., 2004; Valentine et al., 2009; Daud et al., 2013), it was showed that along with either organogenesis or somatic embryogenesis pattern, together with the process of *in vitro* morphogenesis, protein synthesis and catabolism were very active. Also, a number of antioxidant enzyme activities were consistent in the early developmental stages in the two regeneration systems. In view of the results of this study and previous research, it can be assumed that early development mechanisms of both organogenesis and somatic embryogenesis in morphogenesis *in vitro* may be very similar. Further research will help to eventually clarify the molecular mechanisms of plant morphogenesis.

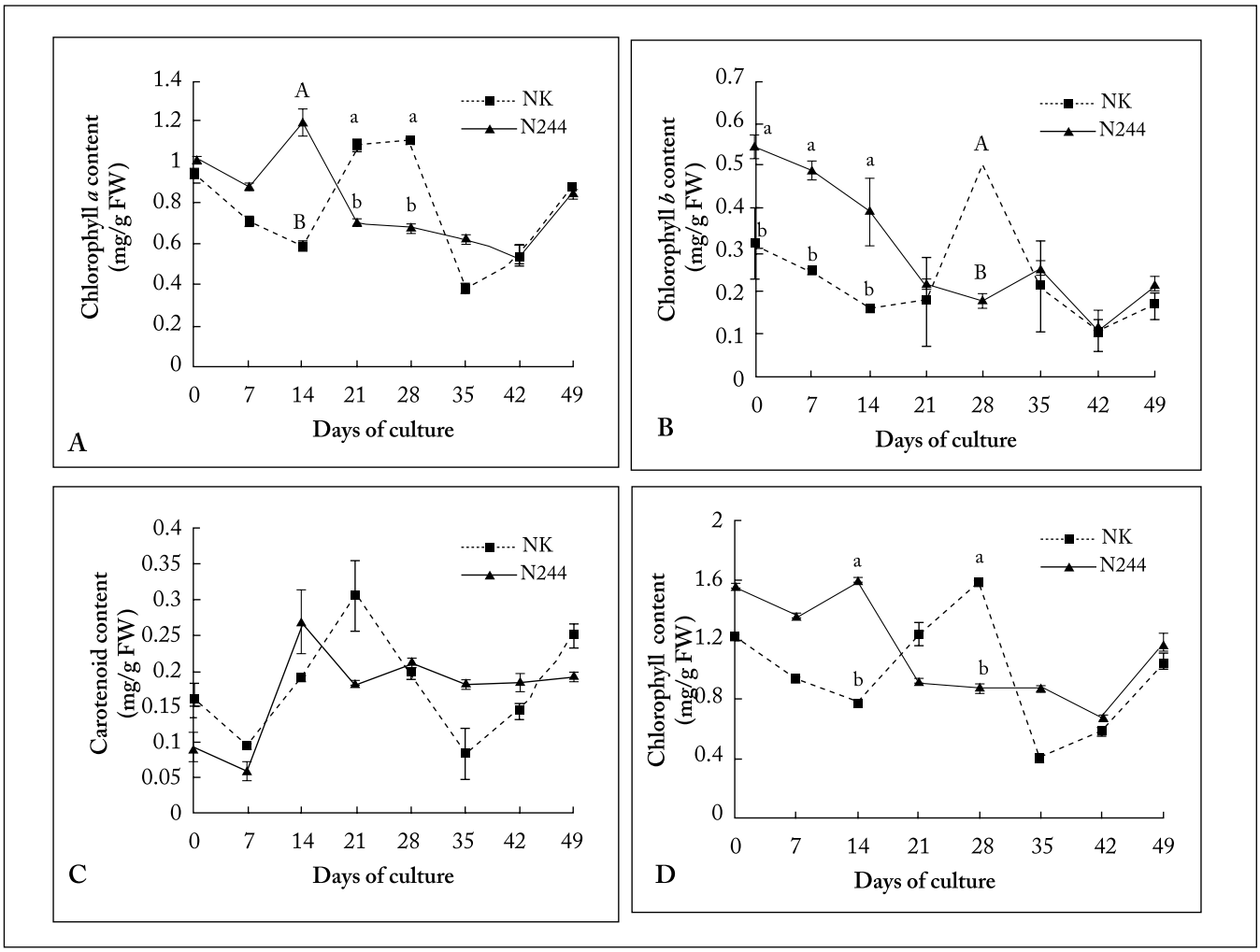

Fig. 2. Changes of chlorophyll *a* content (A), Chlorophyll *b* content (B), Carotenoid content (C) and Chlorophyll content (D) in the callus from both the mutant N244 and the control NK. FW: fresh weight. Mean values and SE were calculated from three independent experiments. Different lowercase letters indicate significant differences at p=0.05. Different uppercase letters indicate significant differences at p=0.01 (Tamhane's multiple range test).

Fig. 2. Cambios en los contenidos de clorofila *a* (A), clorofila *b* (B), Carotenoides (C) y clorofila total (D) en los callos del mutante N244 y del control NK. FW: peso fresco. Los valores promedio y SE fueron calculados de tres experimentos independientes. Letras minúsculas diferentes indican diferencias significativas a p=0,05. Letras mayúsculas diferentes indican valores estadísticamente diferentes a p=0,01 (prueba de comparación de promedios de Tamhane).

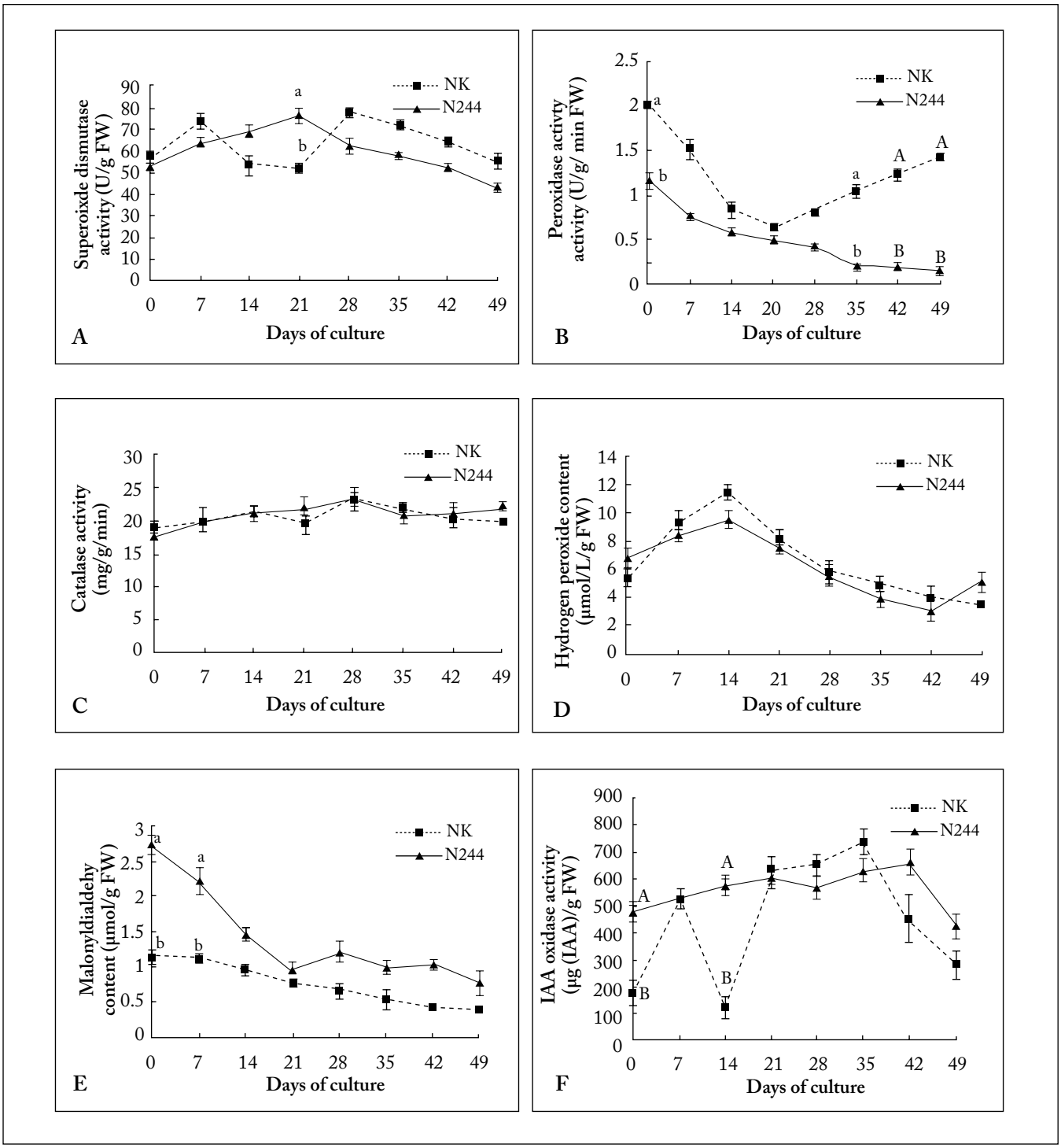

Fig. 3. Changes in level of superoxide dismutase (SOD) (A), peroxidase (POD) (B), and Catalase activities (CAT) (C); H_2O_2 (D) and malondialdehyde (MDA) (E) contents, and IAA oxidase (IAAO) activity (F) in the callus of both the mutant N244 and the control NK. FW: fresh weight. Mean values and SE were calculated from three independent experiments. Different lowercase letters indicate different values at p=0.05. Different uppercase letters indicate different values at p=0.01 (Tamhane's multiple range test).

Fig. 3. Cambios en los niveles de las actividades de la superóxido dismutasa (SOD) (A), peroxidase (POD) (B), y catalase (CAT) (C): contenidos de H_2O_2 (D) y malondialdehido (MDA) (E), y actividad de la IAA oxidase (IAAO) (F) en el callo del mutante N244 y el control NK. Los valores promedio y SE fueron calculados de tres experimentos independientes. Letras minúsculas diferentes indican diferencias significativas a p=0,05. Letras mayúsculas diferentes indican valores estadísticamente diferentes a p=0,01 (prueba de comparación de promedios de Tamhane).

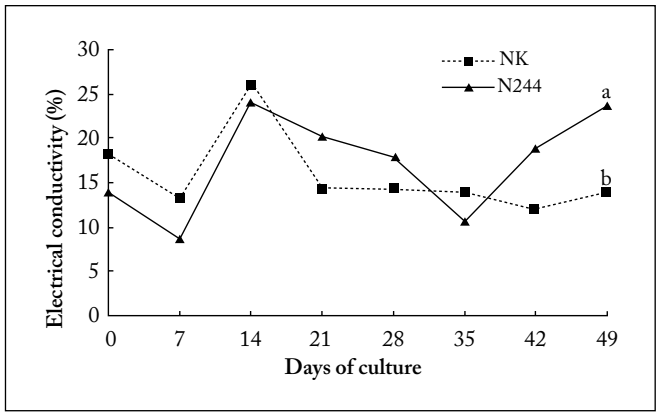

Fig. 4. Changes of electrical conductivity in the callus from both the mutant N244 and the control NK. FW: fresh weight. Mean values and SE were calculated from three independent experiments. Different lowercase letters indicate different values at p=0.05, different uppercase letters indicate different values at p=0.01 (Tamhane's multiple range test).

Fig. 4. Cambios de conductividad eléctrica en los callos del mutante N244 y el control NK. FW: peso fresco. Los valores promedio y SE fueron calculados de tres experimentos independientes. Letras minúsculas diferentes indican diferencias significativas a p=0,05. Letras mayúsculas diferentes indican valores estadísticamente diferentes a p=0,01 (prueba de comparación de promedios de Tamhane).

SOD can regulate the cellular concentration of O_2^- and H_2O_2, and be considered as a key component within the antioxidant defense system. The activities of SOD are usually affected by developmental or environmental factors (Sabir, 2012). CAT can remove a large amount of H_2O_2 (Breusegem et al., 2001). POD was dual-functional, in the ROS metabolism and elimination of H_2O_2, and could induce to produce ROS during pathogen attack (Andrew & Robert, 1997). Other research suggested that a suppressed expression of totipotency in tobacco protoplasts was correlated with a reduced cellular antioxidant activity (Papadakis et al., 2001). This result is similar to that observed in the present study (Fig. 1A and Fig. 3). The activities of the antioxidant enzymes in the mutant callus were much lower than those in the control. This was possibly related to the organogenesis pathway in the mutant callus.

As a rule, cell physiological and biochemical changes always precede the visible changes in morphology. Compared with the control, the HBsAg-transgenic cherry tomato mutant had more significant changes in soluble protein content; chlorophyll *a* and *b* contents, and SOD, POD, and IAA oxidase activities. These changes probably affected the morphogenesis variation of the mutant. Further studies on the developmental morphology variation of the *in vitro* morphogenesis in the transformed cherry tomato mutant would contribute to a better understanding of the developmental morphology mechanisms in the mutant variation.

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