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SHORT COMMUNICATION

Micro-Tuberization as a Long Term Effect of Hydrogen Peroxide on Potato Plants

Humberto A. López-Delgado · Silvia Sánchez-Rojo · Martha E. Mora-Herrera · Ricardo Martínez-Gutierrez

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Abstract Salicylic acid (SA) and hydrogen peroxide (H_2O_2) are involved in similar signaling responses to stress. Previous reports demonstrated micro-tuberization induction by salicylates. Both SA and H_2O_2 induce similar long term signaling responses to stress. In the present work, the potential long term effect of H_2O_2 on the induction of micro-tuberization was evaluated. Nodal explants from micro-plants were immersed in H_2O_2 for 60 min and cultured on medium 30 d. Micro-plants obtained were sub-cultured on tuberization medium and incubated in darkness at 8 and 20±1 C. Pretreatment with 5 and 50 mM H_2O_2 induced the highest micro-tuberization percentage at 20 C, when compared with other pretreatments at 8 C, 1, 5, and 50 mM. H_2O_2 significantly induced more weight per microtuber in all treatments at 20 C. H_2O_2 significantly enhanced sprouting of microtubers produced at 20 C.

H. A. López-Delgado (✉) · S. Sánchez-Rojo · R. Martínez-Gutierrez
Laboratorio de Fisiología-Biotecnología,
Programa Nacional de Papa, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP),
Conjunto SEDAGRO,
Metepec, Estado de México C.P. 52140, México
e-mail: lopez.humberto@inifap.gob.mx

S. Sánchez-Rojo
Facultad de Ciencias,
Universidad Autónoma del Estado de México,
Carr. Toluca-Atlacomulco Km 14.5 El Cerrillo,
Edo, México C.P. 50200, México

M. E. Mora-Herrera
Centro Universitario Tenancingo
Universidad Autónoma del Estado de México,
Carr. Tenancingo-Villa Guerrero Km 1.5,
Edo, México C.P. 52400, México

Resumen El ácido salicílico (SA) y el peróxido de hidrógeno (H_2O_2) están involucrados en respuestas similares de señalización a estrés. Trabajos previos demostraron inducción de microtuberización por salicilatos. Tanto el SA como el H_2O_2 inducen respuestas de señalización a estrés a largo plazo. En el presente trabajo, se evaluó el potencial de inducción a largo plazo del H_2O_2 en la microtuberización. Explantes nodales de microplantas fueron inmersos en H_2O_2 durante 60 minutos y cultivados en medio 30 d. Las microplantas obtenidas se subcultivaron a medio de tuberización y se incubaron en obscuridad a 8 y 20±1 C. A 20 C los pretratamientos con 5 y 50 mM de H_2O_2 indujeron los más altos porcentajes de microtuberización, cuando son comparados con los pretratamientos a 8 C fueron 1,5 y 50 mM. A 20 C H_2O_2 indujo significativamente mayor peso por tubérculo en todos los tratamientos. El H_2O_2 incrementó significativamente la brotación de tubérculos producidos a 20 C.

Keywords Oxygen species · In vitro tuberization

Introduction

The use of microtubers as the final products of potato micro-propagation, in addition to or rather than in vitro plantlets has several advantages in seed tuber production. Microtubers are important in germplasm storage, keeping virus-free germplasm and as a convenient way of exchanging germplasm and they are used as experimental tool in basic research. Their production has been widely studied under different in vitro growing conditions, with varying efficacy regarding the number, size and weight (Estrada et al. 1986;

Dobránszki et al. 2008). They can be produced and stored in the laboratory year-round in order to be directly sown into the soil and produced in bulk in any season without an acclimatization period (Nhut et al. 2004). They have the similar morphological and biochemical characteristics to field produced tubers (Gopal et al. 2004; Zhijun et al. 2005). The number and size of microtubers produced in vitro depend on many factors, such as optimum concentration of sugar, growth regulators and anti-gibberellin compounds in the culture medium (Tovar et al. 1985; Dodds 1988). High sucrose concentrations (60–80 g L^{-1}) promoted biomass and microtuber production as well as microtuber dry matter content (Gopal et al. 2004).

Among the substances used to induce microtubers, coumarin, CCC (Chlorocholide chloride) and cytokinins have received adequate attention (Wang and Hu 1985; Chandra et al. 1988). Cytokinins are believed to have strong promotive effects on tuberization, and may constitute part of the tuberization stimulus, either alone or in combination with other substances like jasmonic acid and abscisic acid (ABA; Palmer and Smith 1969; Pelacho and Mingo-Castel 1991; Sarkar et al. 2006). Growth retardants stimulate tuberization of plants under unfavourable environmental condition (Menzel 1980); CCC as growth retardant is widely used in tissue culture media to promote microtuber formation (Tovar et al. 1985; Rosell et al. 1987).

Growth retardation effects of salicylates in potato micro-plants have been reported (Mora-Herrera et al. 2005). Acetyl salicylic acid (ASA) showed a potent in vitro tuberization-inducing effect when applied as growth retardant in the medium (López-Delgado and Scott 1997; López-Delgado et al. 2007). There are reports associating salicylates and H_2O_2 as endogenous signaling molecules (Scott et al. 1999; Hu et al. 2003; Blee et al. 2004). Hydrogen peroxide (H_2O_2) accumulation can suppress plant growth and development, interfere with different physiological processes, and enhance the response of plants to biotic and abiotic stress conditions (Pnueli et al. 2003). We have demonstrated other potentially useful effects of salicylates on in vitro potato micro-plants such as heat tolerance, growth retardation during storage and virus elimination (López-Delgado and Scott 1997; López-Delgado et al. 1998a, 1998b, 2004, 2007). Considering that SA may be involved in the microtuberization process (Lopez-Delgado and Scott 1997), and both H_2O_2 and SA induce similar signaling responses to stress in potato and growth inhibition among other physiological process (Mora-Herrera et al. 2005; Lopez-Delgado et al. 1998a; Scott et al. 1999) in the present work, an in vitro tuberization procedure was used to evaluate the potential long term effect of H_2O_2 on the induction of micro-tuberization.

Materials and Methods

In Vitro Culture of Stock Micro-Plants

Micro-plants of *Solanum tuberosum* L. cv. Atlantic virus-free from the in vitro Germplasm Bank of the National Potato Program (INIFAP), were propagated from single node cuttings excised and transferred every 4 weeks to fresh MS micro-propagation medium without plant growth regulators (Murashige and Skoog 1962), containing sucrose 3% (*w/v*), and phytagel as a gelling agent, pH 5.7. Plants were cultured in a growth room at 20±1 C under 16 h of photoperiod (fluorescent lights 35 μ mol m^{-2} $s^{-1;}$ 400–700 nm) in sterile conditions (Espinoza et al. 1986).

Micro-Tuberization Induction

Single node cuttings without leaves from four-week old micro-plants were incubated in H_2O_2 (0, 1, 5 and 50 mM) for 60 min and rinsed three times with sterile distilled water. Stem cuttings were cultured on MS medium and incubated under in vitro culture conditions for 30 d. After this period, micro-plants were sub-cultured on an inducing tuberization medium (TM) containing MS salts, thiamine 0.10 mgL^{-1}, myo-inositol 100 mgL^{-1}, 8% (*w/v*) sucrose, benzylaminopurine (BAP) 5 mM and 4.5 g L^{-1}phytagel as a gelling agent. They were incubated in darkness at 8±1 and 20±1°C. Percentage of formed microtubers was recorded on time course every 10 days. Tuber formation was evaluated visually by an apical spherical swelling of the stolon. Weight/tuber and number/plant were estimated 60 d after culture on tuberization medium.

Induction of Sprouting Microtubers

Microtubers formed were sub-cultured on micro-propagation MS medium supplemented with gibberellic acid (GA; 0.05 mg L^{-1}) for 30 d under culture conditions. Percentage of sprouted microtubers was evaluated after 20 days of culture.

Statistical Analysis

Analysis of data was performed with ANOVA and Tukey ($P<0.05$) with Satatgraphics plus 5.0 to define significant differences between treatments. Data are means of three independent experiments, 12–18 explants were used in each

Table 1 Induction of in vitro tuberization by H_2O_2. Data show percentage of micro-plants with tuber formation in course time of 60 d. Single node cuttings were incubated during 60 min on H_2O_2 concentrations followed by culture on MS medium by 30 d. Micro-plants obtained were subcultured in tuberization medium and incubated for 60 d at A) 20 C, B) 8 C. *Significant over control [0]. Data are means ± SE of three experiments (n=36–48) P<0.05

H_2O_2 treatment	Time (d)					
(mM)	10	20	30	40	50	60
A)						
0	0.00±0	40.60±1.07	44.03±1.43	47.26±0.92	82.00±0.18	92.02±0.58
1	0.00±0	79.15±1.35*	81.63±0.19*	86.06±0.75*	87.95±0.45*	91.56±0.65
5	0.00±0	24.07±2.26	30.35±2.98	37.96±1.87	71.83±3.53	96.83±2.10*
50	9.86±0.95*	32.05±0.75	37.89±2.48	50.64±0.64	54.58±0.44	96.96±1.75*
B)						
0	0.0±0	23.70±1.84	41.00±0.57	57.80±0.86	82.76±0.36	100
1	0.0±0	21.50±1.78	38.00±2.56	83.32±0.61*	100±0.0*	100
5	0.0±0	19.80±1.84	37.00±0.69	64.60±1.22*	99.14±0.0*	100
50	0.0±0	34.90±0.69*	67.90±0.61*	86.23±0.49*	100±0.0*	100

H_2O_2 treatment/experiment. For sprouting 11–15 micro-tubers were used.

Results and Discussion

Micro-plants pretreated with 50 mM H_2O_2 at 20 C rapidly induced micro-tuberization 10 days after subculture in tuberization medium (DAST). With this treatment, 9.86% of the plants formed tubers after 10 days which was 20 days before tuberization was observed in the controls (Table 1A). Interestingly, a low H_2O_2 concentration (1 mM) induced the maximum rate of tuberization with 79.15% of tuberized micro-plants after 20 DAST; keeping a significant step wise increase of tuberized micro-plants up 50 DAST. H_2O_2 concentrations of 5 and 50 mM gradually induced tuberization, showing a remarkable percentage enhancement of tuberized micro-plants between 50–60 DAST leading to significant higher percentage of tuberized microplants relative to control at 60 days of culture (Table 1A).

A higher percentage of tuberization was obtained 60 DAST at 8 C relative to 20 C in all treatments (Table 1B). At 8 C, 50 mM H_2O_2 induced a significant 34.9% of tuberized micro-plants at 20 DAST and maintained a significant higher percentage of micro-tuberization relative to the control thereafter. Both 1 and 50 mM H_2O_2 induced a

Table 2 Evaluation of yield of microtubers induced by H_2O_2. Data were recorded at 60 d of culture on tuberization medium. Single node cuttings were incubated during 60 min on H_2O_2 concentrations followed by culture on MS medium by 30 d. Microplants obtained were subcultured in tuberization medium and incubated for 60 d at A) 20 C, B) 8 C. *Significant over control [0]. Data are means ± SE of three experiments (n=36–48) P<0.05

H_2O_2 treatment (mM)	Weight/tuber (mg)	Number/Plant
A)		
0	122.36±2.86	1.43±0.01
1	143.02±3.03*	1.24±0.00
5	126.48±2.48	1.30±0.01
50	127.50±2.88	1.24±0.00
B)		
0	235.20±3.59	1.23±0.03
1	246.38±3.14*	1.00±0.00
5	251.57±3.30*	1.04±0.04
50	246.18±4.32*	1.44±0.01*

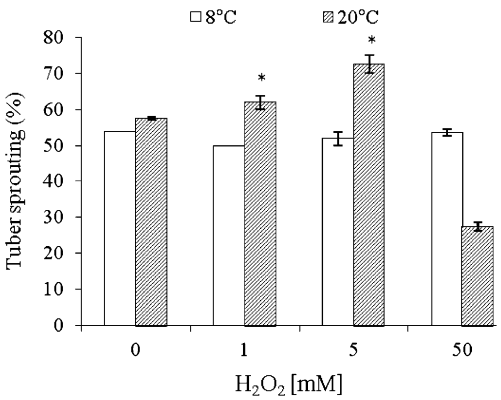

Fig. 1 Sprouting of microtubers induced at 8 and 20 C by H_2O_2. Microtubers formed were subcultured on MS medium added with gibberellic acid (GA; 0.05 mgL^{-1}) for 30 d under culture conditions. Percentage of sprouted microtubers was evaluated after 20 days of culture.*Significant over control [0]. Data are means ± SE of three experiments (n=33–45) P<0.05

significant 100% tuberization 50 DAST; 10 days earlier than control. 1 and 5 mM concentrations significantly induced higher percentage of tuberized micro-plants 40 DAST. After 10 days, 50 mM H_2O_2 significantly enhanced the rate of tuberization of micro-plants.

A high percentage (79.15%) of tuberized micro-plants was obtained 20 DAST at 20 C with a low H_2O_2 concentration (1 mM), suggesting a potential role of H_2O_2 in the signal transduction pathway during tuber induction. Low H_2O_2 concentrations (1, 5 mM) were reported to induce tolerance to stress temperatures in potato, including heat shock (Lopez-Delgado et al. 1998a) and freezing (Mora-Herrera et al. 2005) and these responses to stress were obtained as a long-term effect. In this work a long-term effect of H_2O_2 on morphogenesis in potato tuberization was demonstrated, since short incubation in H_2O_2 for 1 h resulted in a long-term stimulation of tuber induction that persisted for more than 30 days (Table 1A, B).

In contrast with previous reports where culture in tuberization medium was performed immediately after incubation of nodal cuttings in H_2O_2, a higher percentage of tuberized microplants and heavier microtuberswere obtained in the present work (Sanchez-Rojo and Lopez-Delgado 2010). Therefore, subculturing for 30 d on micro-propagation medium after H_2O_2 treatment followed by incubation in tuberization medium increased the tuberization induction effect of H_2O_2.

The known effect of H_2O_2 on growth inhibition (Schopfer 1996; Lopez-Delgado et al. 1998a; Sanchez-Rojo and Lopez-Delgado 2010) could be an important component of the inducing effect of H_2O_2 on tuberization, since tuberization evolves two important processes: growth inhibition of the stolon apical meristem and radial expansion of the sub-apical tuber tissues (García-Torres and Gómez-Campo 1973). Tuberization induction by H_2O_2 occurred in absence of a growth inhibitor such as CCC, which are generally used in some tuberization media (Tovar et al. 1985).

At 8°C, 1 mM H_2O_2 induced a significant increase in microtuber weight relative to controls. No significant differences in the number of microtubers were observed (Table 2B), whereas at 20°C, all H_2O_2 concentrations induced significantly higher weight and for 50 mM a higher number of microtubers as well (Table 2A). It is likely that the H_2O_2 concentration effect is influenced by temperature, since the physiological age of microplants was the same at both temperatures.

The increased weight of microtubers following H_2O_2 treatment could be associated with more starch accumulation, since 5 and 50 mM H_2O_2 enhanced starch accumulation in potato plants sprayed under field conditions (Lopez-Delgado et al. 2005) and in phytoplasma-positive potato plants treated with 1 mM H_2O_2 under greenhouse conditions (Romero-Romero and Lopez-Delgado 2009). Temperature is one of the major environmental factors affecting potato tuberization. Cold temperatures are very favorable for tuber induction, while high temperatures exert a negative effect (Ewing and Struik 1992). It was demonstrated that low temperatures (4°C) delayed tuberization but enhanced the weight of tubers (Martinez and Tizio 1990). In the present work, low temperature (8°C) reduced the weight and percentage of tuberized micro-plants in contrast to high temperature(20°C).

The main problems associated with microtuber production are low yield and the small size of tubers that limits direct transplanting to the field (Jiménez et al. 1999). For practical purposes, the effect of H_2O_2 on microtuber weight is significant as it was suggested that microtuber weight is more important than microtuber number in determining microtuber yield potential (Uranbey et al. 2004).

Temperature during tuberization induction was an important factor for the subsequent induction of sprouting. No significant differences were observed on sprouting in microtubers produced at 8°C (Fig. 1). However, tuberization induction at 20°C had positive effects on sprouting. One and 5 mM H_2O_2 stimulated a significantly higher percentage of sprouting comparing the control with 5 mM producing the greatest effect (72.5%vs. 57.5% in controls; Fig. 1). Conversely, 50 mM H_2O_2 significantly inhibited the sprouting (27.4%). Therefore, the H_2O_2 effect on sprouting was significantly different depending on the temperature of tuberization induction. These results are in agreement with previous reports suggesting low temperatures during tuber formation, can extend significantly the dormancy of the microtubers (Thieme 1992;Martinez and Tizio 1990), probably by enhancing the ABA content as suggested by van den Berg et al. (1991).

The potential long term effect of H_2O_2 on in vitro tuberization and the practical utility of this treatment is worthy of further research.

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