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Short communication. N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU) enhances *in vitro* direct shoot organogenesis of *Citrus aurantium* L. epicotyl segments compared to other commonly used cytokinins

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Abstract

The effect of three concentrations of five different cytokinins, *i.e.* 6-benzylamino purine, 2-isopentyl adenine, kinetin (Kin), thidiazuron and N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU), was evaluated on the *in vitro* direct shoot organogenesis of epicotyl explants of sour orange (*Citrus aurantium* L.). The basal medium used was that of Murashige and Tucker and epicotyl explants were incubated in medium supplemented with the prementioned cytokinins for 45 days. The addition of Kin and 4-CPPU in the medium enhanced the direct shoot organogenesis of sour orange epicotyl segments. The concentration of each of these two cytokinins which gave the best results, was combined with indole-3-acetic acid (IAA) or α-naphthalene acetic acid (α-NAA) at concentrations ranging from 0.01 mg L^{-1} to 0.2 mg L^{-1}. The inclusion of IAA at 0.2 mg L^{-1} in the medium with 4-CPPU at 0.05 mg L^{-1} resulted in 100% successful direct shoot organogenesis, while the combination of Kin at 0.25 mg L^{-1} with IAA or α-NAA each at 0.01 mg L^{-1} presented equally high organogenesis percentages (91.7%). The incubation of the produced shoots, in medium supplemented with either indole-3-butyric acid or α-NAA resulted in high rooting percentages (up to 90%) and the rooted explants were successfully acclimatized under mist (85%). Although 4-CPPU has been used in *in vitro* culture of various species, this is the first report on its use in the direct shoot organogenesis of citrus species and could be of great value in citrus genetic transformation protocols using epicotyl segments, since this cytokinin resulted in the absolute organogenesis percentage.

Additional key words: 2-isopentyl adenine; 6-benzylamino purine; explants; kinetin; sour orange; thidiazuron.

Resumen

Comunicación corta. El N-(2-cloro-4-piridil)-N-fenilurea (4 CPPU) mejora la regeneración *in vitro* vía organogénesis directa de segmentos de epicótilo de *Citrus aurantium* L. en comparación con otras citoquininas de uso común

Se evaluó el efecto de tres concentraciones de cinco diferentes citoquininas [6-bencilaminopurina; 2-isopentil adenina; kinetina (Kin), tidiazurón y N-(2-cloro-4-piridil)-N-fenilurea (4-CPPU)], en la organogénesis directa *in vitro* de explantes de epicótilo de naranjo amargo (*Citrus aurantium* L.). Se incubaron durante 45 días explantes de epicótilo en medio Murashige y Tucker suplementado con las cinco citoquininas. La adición de Kin y 4-CPPU en el medio aumentó la organogénesis directa de segmentos de epicótilo de naranjo amargo. Se combinó la concentración que dio mejores resultados de cada uno de estas dos citoquininas con ácido indol-3-acético (IAA) o con ácido α-naftaleno acético (α-NAA) en concentraciones desde 0,01 hasta 0,2 mg L^{-1}. La inclusión de IAA a 0,2 mg L^{-1} en el medio con 4-CPPU a 0,05 mg L^{-1} resultó en un 100% de éxito en la organogénesis directa, mientras que la combinación de Kin a 0,25 mg L^{-1} con IAA o α-NAA, ambas a 0,01 mg L^{-1}, resultó en un 91,7% de organogénesis. La incubación de los

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Abbreviations used: α-NAA (α-naphthalene acetic acid); 2iP (2-isopentyl adenine); 4-CPPU (N-(2-chloro-4-pyridyl)-N-phenylurea); BA (6-benzyladenine); CTV (*Citrus tristeza* virus); IAA (indole-3-acetic acid); IBA (indole-3-butyric acid); Kin (kinetin); MT (Murashige and Tucker basal growth medium); TDZ (thidiazuron).

brotes producidos en medio suplementado con ácido indol-3-butírico ó α-NAA resultó en altos porcentajes de enraizamiento (hasta un 90%) y los explantes enraizados se aclimataron con éxito (85%) en un *mist.* Se ha utilizado el 4-CPPU en el cultivo *in vitro* de diferentes especies, pero este es el primer informe sobre su uso en la organogénesis directa de cítricos y podría ser de gran valor en los protocolos de transformación genética de segmentos de epicótilo de cítricos, ya que esta citoquinina produjo un 100% de organogénesis.

Palabras clave adicionales: 2-isopentil adenina; 6-bencilaminopurina; explantes; kinetina, naranjo amargo; tidiazurón.

The usefulness of rootstocks in fruiting tree cultivation is undoubtedly significant. Rootstock significance is becoming even greater for some species, such as *Citrus* spp., where they are used against unfavorable pedoclimatic conditions and biotic stress factors. Some of these rootstocks are propagated by sowing open-pollinated seeds, which produce highly nucellar true-to-type plants (Sharma *et al*., 2009). At the same time, polyembryony, sexual incompatibility and male or female sterility hamper the improvement of citrus by conventional method (Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Duan *et al*., 2007). Sour orange (*Citrus aurantium* L.) is one of the most popular rootstocks used in citriculture, due to its great properties (tolerance) against root rot and calcareous soils and the high fruit quality of grafted cultivars. Unfortunately, the susceptibility of sour orange combinations with mandarin (*Citrus reticulata* Blanco) and orange [*Citrus sinensis* (L) Osbeck] to *Citrus tristeza virus* (CTV) has lead to a significant reduction of sour orange use as a rootstock and to a promotion of the use of more tolerant trifoliate hybrids. However, these hybrids may be tolerant to CTV but they are susceptible to calcareous soils and some viroids while their combination with citrus species may produce low quality fruits. One possible solution would be the genetic transformation of sour orange to induce tolerance to CTV. Among rootstocks, transgenic plants have easily been obtained for Carrizo citrange (Peña *et al*., 1995), however, other rootstocks, such as *C. limonia*, *C. volkameriana* and sour orange, proved to be recalcitrant (Gutiérrez *et al*., 1997; Azevedo *et al*., 2006). These difficulties may be related not only to gene transfer process but also to *in vitro* organogenesis (Tavano *et al*., 2009). The low *in vitro* organogenesis rate results in low genetic transformation efficiency. In order to establish an efficient *in vitro* organogenesis protocol, the effect of various cytokinins, one of them not commonly used, was studied on adventitious shoot development in sour orange epicotyl segments.

Mature sour orange fruit were collected and transferred to the laboratory. The seeds were collected, washed under running tap water for 15 min and blotted dry with a paper towel. Special care was taken during seed coatings removal, in order to avoid seed wounding. When a sufficient number of seeds were gathered, the peeled seeds were disinfected with 60% v/v sodium hypochlorite for 10 min and washed at least three times with sterile distilled water. The seeds were then transferred to test tubes (one seed per tube) containing 10 mL of Murashige and Tucker (MT) (Murashige and Tucker, 1969) basal growth medium, pH 5.2, supplemented with 30 g of sucrose. The seeds were then incubated under dark for 30 days at 25°C and later under 16/8 h photoperiod under cool white fluorescent lamps (38 μmol m^{-2} s^{-1}) for another 15 days. Epicotyl segments were aseptically transversally cut and transferred horizontally in MT basal medium in Petri dishes, supplemented with the following cytokinins: 6-benzyl adenine (BA), kinetin (Kin) and 2-isopentyl-adenine (2iP) at concentrations 0.25, 0.5, and 1.0 mg L^{-1} each and thidiazuron (TDZ) and N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU) at concentrations of 0.05, 0.1 and 0.2 mg L^{-1} (filter sterilized). A medium non-supplemented with cytokinin served as control treatment. The explants remained in the pre-mentioned media for 45 days under 16/8h light/dark photoperiod. At the end of that period the percentage of shoot organogenesis, the number and length of shoots as well as the presence of callus mass was measured. In order to increase the organogenesis percentages, the concentration of the two cytokinins which resulted in the highest organogenesis percentages was combined with the auxins indole-3-acetic acid (IAA) and α-naphthalene acetic acid (α-NAA) at concentrations of 0.01, 0.05, 0.1 and 0.2 mg L^{-1}. At the end of the 45 day period the same variables were evaluated, as previously described. The shoots produced were then transferred into MT basal medium supplemented with the auxin indole-3-butyric acid (IBA) or α-NAA at concentrations of 1 mg L^{-1}, in order to induce rhizogenesis. Rooting percentages were recorded after 6 weeks. Rooted explants were transferred to a mist irrigation system for acclimatization. Plantlets which continued to grow after 4 weeks under mist were considered as acclimatized.

The trial was repeated two times and followed the completely randomized design, with four replications. Data concerning the effect of cytokinins on organogenesis were analyzed as a multifactor Anova, with the two factors being the cytokinin used and its concentration (classified as low, medium and high). Significant differences between means were detected through the standard error bars (of the MANOVA analysis) within each graph. The means of each treatment were also compared with the control treatment by the Dunnett's test (Koenig *et al.*, 2008) at $\alpha = 0.05$. Data from the combination of cytokinins and auxins were analyzed as an one-way Anova and differences were compared with the Tukey HSD test at $\alpha = 0.05$.

The cytokinin used had a significant effect on the organogenesis percentage ($p < 0.001$), on the mean shoot number ($p < 0.05$) and their mean length ($p < 0.05$) (Fig. 1). The concentration of the cytokinin used had a significant effect only on the organogenesis percentage achieved ($p < 0.01$), as every cytokinin tested proved to be more efficient at its lowest concentration. The inhibitory effect of high cytokinin concentration (especially that of BA) has been reported with other citrus species too (Moreira-Dias *et al.,* 2000; Molina *et al.,* 2007; Cervera *et al.,* 2008). The control treatment resulted in nearly 5% successful organogenesis. The addition of 2iP in the medium, resulted in the highest percentage of callus production in the cut ends (20-55% of explants, depending on the concentration used), followed by that of TDZ (10-55%). The addition of BA, 4-CPPU and Kin resulted in the lowest callus production (20-30%, 20-30% and 5-25%, respectively) (Fig. 2). Among the cytokinins used, BA, 4-CPPU and Kin resulted in the highest organogenesis percentages, while the explants did not respond to the addition of either TDZ or 2iP in the medium, as has been reported on other citrus species too (Pérez-Molphe-Balch and Ochoa-Alejo, 1997). The former three cytokinins, at their two lowest concentrations, gave significantly better results than that of the control, as indicated by Dunnett's test. The low responsiveness to the addition of 2iP in the medium could be ascribed to genotype preference, along with 2iP sensitivity to cytokinin oxidases, due to the double bond in the molecule (Arinaitwe *et al.,* 2000). BA is the cytokinin mostly used in the direct citrus epicotyl organogenesis, with similar results to ours (Bordón *et al.,* 2000; Costa *et al.,* 2004; Germana *et al.,* 2008). Kinetin has been sporadically used, as the percentages achieved were usually lower than that of BA, although values of 70% successful organogenesis have been reported for rough lemon, Cleopatra mandarin and *C. depressa* (Sharma *et al.,* 2009). The positive effect of Kin addition could be related to the genotype used. To our knowledge, 4-CPPU has not been tested in the *in vitro* citrus shoot organogenesis technique, although there are reports on its use in citrus embryogenesis (Siragusa *et al.,* 2007) as well as in other species, especially during the proliferation stage (shoot regeneration) (Arinaitwe *et al.,* 2000; Vinayak

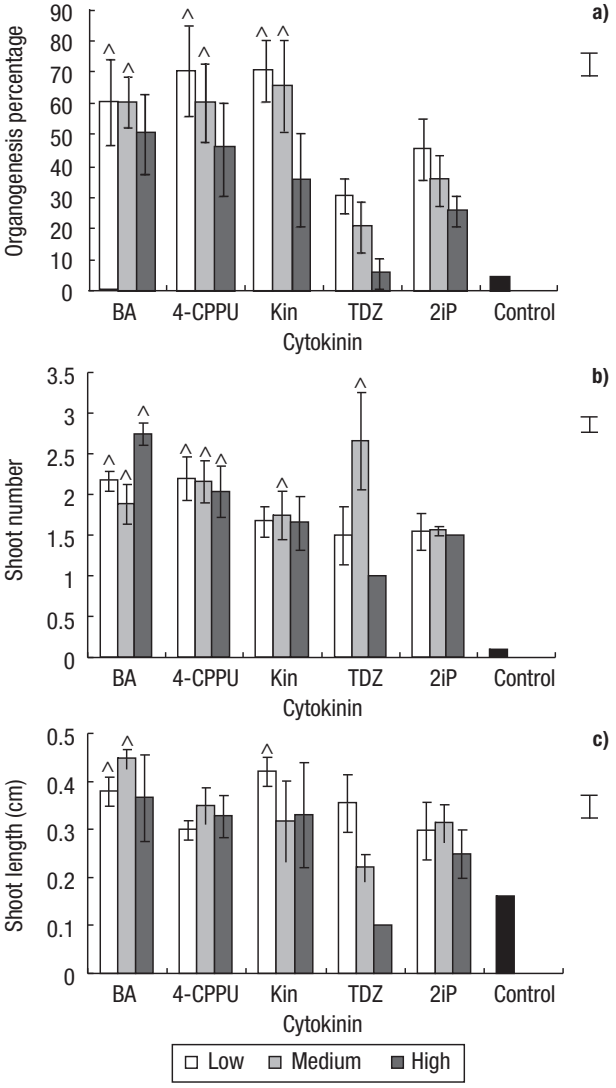

Figure 1. Effects of cytokinins [BA (6-benzyladenine); 4-CPPU (N-(2-chloro-4-pyridyl)-N-phenylurea); Kin (kinetin); TDZ (thidiazuron); 2iP (2-isopentyl adenine)] and their concentrations on the direct organogenesis percentage (a), on the mean shoot number per explant (b) and on the mean shoot length (c). Bars on each column represent the standard deviation of the mean. Bars at the right side of the graphs are the standard error of the Multifactor Anova. Symbols (^) above the columns indicate significant difference of the specific treatment from control, based on Dunnett's test.

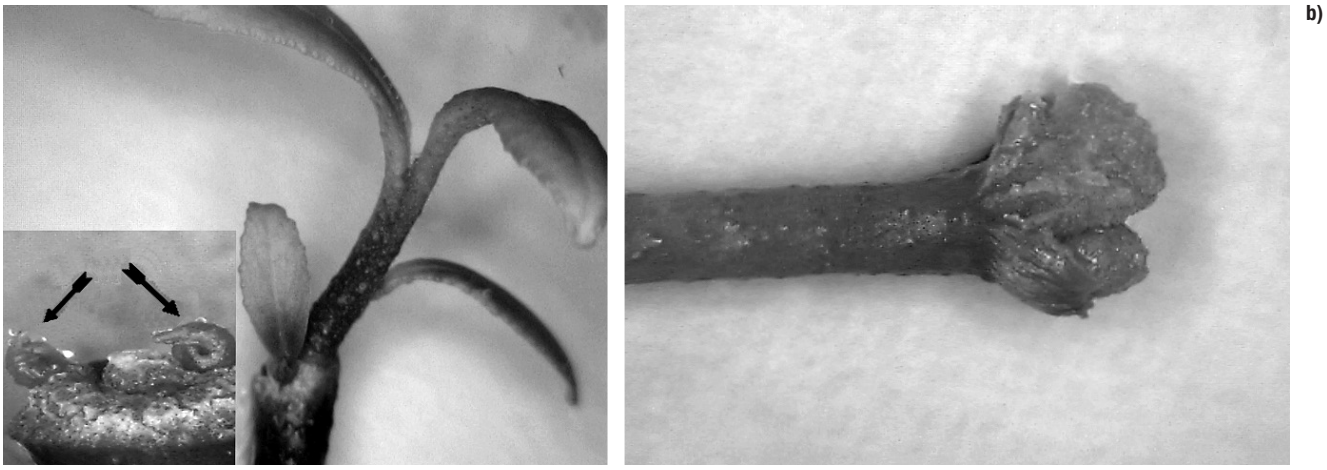

Figure 2. Direct *in vitro* shoot organogenesis in epicotyl segment of sour orange (a) direct bud (bottom left, arrows indicating bud development) and shoot formation in the presence of N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU), (b) callus formation in the presence of 2-isopentyl adenine (2iP).

et al., 2009). The 4-CPPU is a phenylurea, as is TDZ, which is very effective even at very low concentrations, basically due to phenylureas' relative tolerance to endogenous cytokinin oxidases (Arinaitwe *et al.,* 2000), along with the ability to promote endogenous cytokinin production, through the inhibition of cytokinin oxidases activity (Magioli *et al.,* 1998). The differences observed between the two phenylureas could be attributed to the genotype responsiveness, as it has also been reported for adenine type cytokinins (Bordón *et al.,* 2000). The addition of 4-CPPU along with that of BA resulted in the highest number of shoots produced, while Kin and BA had a significant effect on the mean shoot length. The addition of IAA at 0.2 mg L^{-1} in combination with 4-CPPU at 0.05 mg L^{-1} resulted in the highest organogenesis percentage of 100% (Table 1). High organoge-

Table 1. Efficiency of the combination of cytokinins [kinetin (Kin) and N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU)], plus auxins [indole-3-acetic acid (IAA) and α-naphthalene acetic acid (α-NAA)] on the direct shoot organogenesis of sour orange epicotyl explants

Treatments (mg L^{-1})				Organogenesis (%)	Mean shoot number	Mean shoot length (cm)
4-CPPU	Kin	IAA	α-NAA			
0.05	—	—	—	62.5 ± 12.5^{ab}	2.4 ± 0.2^{b}	0.51 ± 0.06^{a}
0.05	—	0.01	—	12.5 ± 12.5^{a}	1.8 ± 0.0^{ab}	0.48 ± 0.01^{a}
0.05	—	0.05	—	12.5 ± 12.5^{a}	2.0 ± 0.0^{ab}	0.40 ± 0.01^{a}
0.05	—	0.1	—	37.5 ± 24.0^{ab}	2.0 ± 0.0^{ab}	0.35 ± 0.05^{a}
0.05	—	0.2	—	100 ± 0.0^{b}	1.6 ± 0.1^{ab}	0.42 ± 0.07^{a}
0.05	—	—	0.01	37.5 ± 24.0^{ab}	2.0 ± 1.0^{ab}	0.50 ± 0.10^{a}
0.05	—	—	0.05	75 ± 14.0^{ab}	1.5 ± 0.3^{ab}	0.35 ± 0.06^{a}
0.05	—	—	0.1	50 ± 20.4^{ab}	1.3 ± 0.3^{ab}	0.35 ± 0.03^{a}
0.05	—	—	0.2	$37.5 \pm^{ab}$	1.5 ± 0.5^{ab}	0.62 ± 0.32^{a}
—	0.25	—	—	66.7 ± 8.3^{ab}	1.4 ± 0.2^{ab}	0.41 ± 0.07^{a}
—	0.25	0.01	—	91.7 ± 8.3^{ab}	1.3 ± 0.2^{ab}	0.53 ± 0.06^{a}
—	0.25	0.05	—	83.3 ± 8.3^{ab}	1.1 ± 0.1^{ab}	0.58 ± 0.17^{a}
—	0.25	0.1	—	66.7 ± 22.0^{ab}	1.1 ± 0.1^{ab}	0.43 ± 0.12^{a}
—	0.25	0.2	—	83.3 ± 8.3^{ab}	1.5 ± 0.2^{ab}	0.51 ± 0.07^{a}
—	0.25	—	0.01	91.7 ± 8.3^{ab}	1.3 ± 0.1^{ab}	0.73 ± 0.22^{a}
—	0.25	—	0.05	83.3 ± 8.3^{ab}	1.1 ± 0.1^{ab}	0.54 ± 0.09^{a}
—	0.25	—	0.1	58.3 ± 8.3^{ab}	1.0 ± 0.0^{a}	0.71 ± 0.19^{a}
—	0.25	—	0.2	75.0 ± 0.0^{ab}	1.2 ± 0.2^{ab}	0.43 ± 0.09^{a}

Means within the same column followed by the same letter do not differ significantly by the Tukey HSD test, at $\alpha = 0.05$.

nesis percentages were also observed when Kin, at 0.25 mg L^{-1}, was combined with either IAA or α-NAA at 0.01 mg L^{-1}. The combination of auxins and cytokinins has proved to be more efficient in inducing organogenesis in various citrus species, compared to cytokinin alone (Pérez-Molphe-Balch and Ochoa-Alejo, 1997; Duan *et al.,* 2007), although the use of IAA has not been extensively investigated (Duan *et al.,* 2007). Shoot length did not differ among treatments, while the shoot number was the highest, when 4-CPPU was used alone and lowest when Kin was combined with 0.1 mg L^{-1} α-NAA. When the shoots were excised and cultured in medium supplemented with either IBA or α-NAA, the rooting percentages reached 90% and 75%, respectively, while the rooted explants were successfully acclimatized under mist (85% successful acclimatization). The responsiveness of citrus epicotyl explants to direct organogenesis has been reported to range from very high percentages, such as 100% for grapefruit (Costa *et al.,* 2004), 90-95% for Troyer and Carrizo citranges (Bordon *et al.,* 2000; Moreira-Dias *et al.,* 2000) to values around 70% for sweet orange (Duran-Vila *et al.,* 1992). However some genotypes seem to be very recalcitrant, exhibiting very low organogenesis efficiency, such as 45 to 60% for *C. limonia* (Costa *et al.,* 2004) to the extreme low responsiveness of 4% for sour orange (Bordon *et al.,* 2000). In the present experiment, epicotyl explants responded successfully to the combination of 4-CPPU and IAA, achieving the absolute of 100% successful organogenesis. The use of 4-CPPU has not been reported in citrus epicotyl explant direct shoot organogenesis, although it has been used in citrus embryogenesis and could be a valuable alternative to the use of BA for other genotypes too.

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