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Original artícle

Effect of explant and picloram on embryogenic callus formation in 'Blanco de Guinea' yam

Efecto del explante y el picloram en la formación de callos embriogénicos en ñame *(Dioscorea cayenensis* subsp rotundata Poir), var. 'Blanco de Guinea'

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ABSTRACT: The shortage of planting material of physiological and sanitary quality continues to limit the large-scale production of yam cultivation since the tubers, which constitute the useful part of the plant for food, also have to be used as planting plant material. The work was developed with the objective of evaluating the explant type effect and the concentration of picloram in the formation of embryogenic callus in *Dioscorea cayenensis* subsp rotundata cultivar Blanco de Guinea. Nodal segments, immature leaf and root segments from *in vitro* plants were taken as explants. The culture medium for callus formation contained the DM salts and different concentrations of Picloram (0; 0.5; 1.0; 1.5; 2.0 mg L^{-1}). The callus formation percentage was determined for each type of explant, the color and consistency of the callus. It was achieved with the use of 2.0 mg L^{-1} of Picloram, 80% of nodal segments with embryogenic callus formation, yellowish-brown in color, compact and friable.

Keywords: auxins, tissue culture, Dioscorea, *in vitro*.

RESUMEN: La escasez de material vegetal de plantación con calidad fisiológica y sanitaria continúa limitando la producción a gran escala del cultivo del ñame ya que los tubérculos, que constituyen la parte útil de la planta para la alimentación, también tienen que ser utilizados como material vegetal de plantación. El trabajo se desarrolló con el objetivo de evaluar el efecto del tipo de explante y la concentración de Picloram en la formación de callos embriogénicos en *Dioscorea cayenensis* subsp rotundata var. 'Blanco de Guinea'. Se tomaron como explantes, segmentos nodales, hojas inmaduras y segmentos de raíces provenientes de plantas *in vitro*. El medio de cultivo para la formación de callos contenía las sales MS y diferentes concentraciones de Picloram (0; 0,5; 1,0; 1,5; 2,0 mg L^{-1}). Se determinó el porcentaje de formación de callos para cada tipo de explante, el color y consistencia de los callos. Se alcanzó con empleo de 2,0 mg L^{-1} de Picloram, un 80 % de segmentos nodales con formación de callos embriogénicos, de color pardo-amarillento, consistencia compacta y friables.

Palabras clave: auxinas, cultivo de tejidos, Dioscorea, *in vitro*.

INTRODUCTION

Yam (*Dioscorea* spp.) occupies the fourth place in the world production of roots and tubers, after potato (*Solanum tuberosum* L.), cassava (*Manihot esculentaCrantz*) and sweet potato (*Ipomoea batatas* L.) (1); besides, there are more than 600 species among which *D. alata* and *D. rotundata* stand out (2).

In this crop there is progress in the production of seeds by traditional methods (3) and their conservation by biotechnological methods (4), but its extensive development has been limited by the scarcity of plant material with good physiological and sanitary quality (5), due to the fact that the tubers are used, both for food and as starting material for planting (6), and losses reach up to 50 % during post-harvest and storage, due to the susceptibility of tubers to bacteria, nematodes and fungi such as *Colletotrichum gloeosporioides* (7).

Similarly, the application of genetic engineering has been limited by the absence of efficient protocols that allow the

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regeneration of plants and their genetic transformation (5). An alternative is somatic embryogenesis, a process by which somatic cells can develop somatic embryos into plants (8).

Internationally, progress in somatic embryogenesis in yam has been described in a small number of species and cultivars (5), with low frequencies of somatic embryo induction and variations among genotypes (9); however, the conversion of somatic embryos to plants has not been efficiently achieved (10).

In *Dioscorea rotundata* there are limitations in the different phases of somatic embryogenesis, such as induction percentages of pro-embryogenic masses lower than 30 %, low percentages of plant regeneration and protocols not reproducible in other genotypes (5,7,9).

In Cuba, plant regeneration via somatic embryogenesis was possible in the cultivar 'Blanco de Guinea' (6,11), but only 6.0 % of the callus obtained from leaves with petioles, were able to form embryogenic structures when they were cultivated with 2,4-dichlorophenoxyacetic acid (2,4-D) and callogenesis was not observed in root sections, being necessary new studies that allow increasing this response.

For this reason, the present work was aimed at determining the effect of explant type and Picloram concentration on callus formation in yam (*Dioscorea cayenensis* subsp rotundata Poir) 'Blanco de Guinea'.

MATERIALS AND METHODS

The research was conducted at the Center for Plant Biotechnology Studies (CEBVEG) of the University of Granma (UDG), located in Peralejo, Bayamo, Granma, Cuba.

Plant material

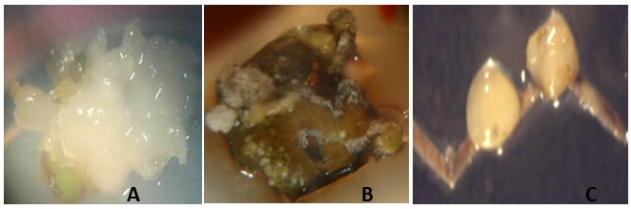
Plants of *Dioscorea cayenensis* subsp rotundata Poir 'Guinea white', were maintained and multiplied *in vitro* from nodal segments, grown on the culture medium, containing the salts and vitamins MS (12) 4.41g L^{-1}, 6-benzylaminopurine 0.05 mg L^{-1}, naphthaleneacetic acid 0.02 mg L^{-1}, ascorbic acid 25 mg L^{-1}, sucrose 30 g L^{-1} and Gelrite 2.4 g L^{-1}.

Effect of explant type

With the purpose of inducing the formation of callus with embryogenic structures, the effect of different types of explants (nodal segments, root segments and immature leaves placed on the culture medium in adaxial/abaxial position), coming from *in vitro* 'Blanco de Guinea' yam plants grown for five weeks, was determined.

The different types of explants were placed in a culture medium with complete salts including vitamins MS (12) 4.41 g L^{-1}; sucrose 30 g L^{-1}, ascorbic acid 25 mg L^{-1}, Picloram 1.0 mg L^{-1}, pH 5.8 and Gelrite® 2.4 g L^{-1}.

To determine the onset of callus formation, weekly observations were made with a stereoscopic microscope (Olympus, 10x) and the color and consistency of the callus was evaluated; the percentage of callus formation was determined after 30 days of culture.



A) Embryogenic callus from nodal segments, B) Immature leaves in abaxial position C) Root segments with somatic embryos

Figure 1. Callus-forming explants on *Dioscorea rotundata var.* rotundata var. 'Blanco de Guinea' grown in the dark with Picloram with Picloram 1.0 mg L^{-1} for 30 days

Effect of Picloram concentration

Subsequently, the best type of explant determined in the previous experiment grown in different concentrations of Picloram (0.5; 1.0; 1.5; 2.0mg L^{-1}) including a control treatment without growth regulator was used.

Twenty-five replicates per treatment were used and placed in glass flasks containing 10 mL of culture medium for four weeks of culture. These were placed in dark conditions at a temperature of 25±2 °C. At 30 days of culture, the number of explants with embryogenic callus formation (%) was determined.

Statistical analysis

To verify whether the data complied with the assumptions of normality of the data, the Shapiro Wilks test was used and for homogeneity of variance the Kolmogorov-Smirnov test was used.

The percentage of callus formation (%) by explant type was determined according to the mathematical expression: % FC=Total explants with callus/Total explants X 100.

Qualitative variables were analyzed using descriptive statistics and data expressed as percentages were analyzed using a comparison of proportions analysis with the CompraPro statistical package.

RESULTS ANDY DISCUSSION

Effect of explant type

In all explants grown with Picloram 1.0 mg L^{-1}, phenolization of the plant tissue and thickening of the axillary bud of the nodal segments were observed from the second week of culture (Figure 1).

Subsequently, there was an abundant proliferation of white callus with nodular structures, compact and friable consistency (Figure 1A); when immature leaves were used, they curved and formed callus on the explant edges and on the main rib of the leaf blade (Figure 1B); meanwhile, in the root segments there was scarce callus formation with a tendency to the formation of isolated somatic embryos and in a direct way (Figure 1C).

The white callus observed in the present investigation, are comparable with those described by other authors, who made reference to the presence of non embryogenic callus similar to cotton specks in *Dioscorea rotundata* (5), in other studies have been described callus of crystalline and filamentous appearance, in some cases associated with pro-embryogenic masses, which were formed by non embryogenic cells of elongated form, devoid of nucleus and little cytoplasmatic content (7).

The explants showed differential responses to callus formation in the presence of Picloram 1.0 mg L^{-1}. The highest response was obtained in about half of the explant nodal segments used, with significant statistical differences compared to the values obtained in the rest of the explant types used (Figure 2).

Differences were observed according to the abaxial and adaxial orientation of the leaves on the culture medium. No callus formation occurred on the adaxial side of the leaves; on the other hand, the abaxial side of the leaves and the root segments responded with percentages lower than 7.0 % of callus formation and without significant statistical differences between these types of explants (Figure 2).

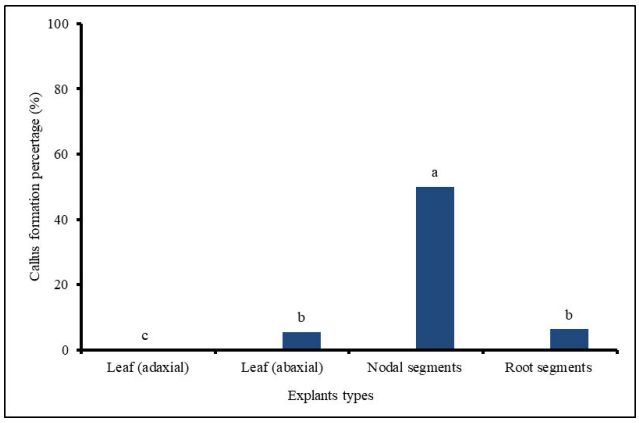
The response observed from root segments differs from that reported in the scientific literature (11), where callogenesis was not observed in this type of explant and the same genotype but with 2.0 mg L^{-1} of 2.4-dichlorophenoxyacetic acid.

In addition, it could be related to the fact that roots are the main site of cytokinin synthesis in plants (13), and whose endogenous concentration when interacting with the auxin added to the culture medium, propitiated an adequate hormonal balance for the occurrence of somatic embryogenesis.

The differences in the response of the leaves according to their orientation on the culture medium could be linked to the presence of different types of cells in the epidermis of both sides of the leaves. A study on the differences in leaf architecture of three species, *Dioscorea* sp, *D. glomerulatay D. haumanii*, revealed that all have adaxial epidermis with isodiametric cells with straight to curved walls; the abaxial epidermis has abundant stomata and isodiametric cells except in Dioscorea sp. where the cells were rectangular (14).

Meanwhile, somatic embryogenesis has been reported in *D. rotundata* var. 'Blanco de Guinea' using explants of leaves with petioles obtained from plants *in vitro*. At two weeks, the formation of proembryogenic masses was observed with the highest presence in the 1.0 mg L^{-1} picloram treatment; the use of sucrose increased the development of somatic embryos and favored conversion to plant (15).

The nodal segments showed a better response for callus formation, this result may be due to the fact that, when the axillary bud is in the nodal segment, there is a greater quantity of reserve substances and meristematic tissue, therefore, when adding Picloram it stimulated the callus formation process, since auxins have action on cellular elongation, tissue expansion, cellular division and formation of adventitious roots.



Different letters on the bars indicate significant statistical differences according to comparison of proportions (p≤0.05)

Figure 2. Callus formation response in different types of explants with 1.0 mg L^{-1} of Picloram at 30 days of culture

The results with the use of nodal segments are comparable with those obtained by other researchers in this same species (7), but with the use of another type of explant, who used leaves with petiole and obtained more than 66 % callus formation under the effect of Picloram 2.0mg $^{L-1}$ in *D. rotundata* cultivar Aleman.

These results indicate the need for proper selection of explant type for callus induction and somatic embryogenesis, particularly in monocotyledonous plant species, where cells differentiate rapidly, followed by loss of mitotic and morphogenetic capacity.

Effect of Picloram concentration

Explants from nodal segments grown in various concentrations of Picloram showed differential responses to callus formation, with significant statistical differences among treatments. Callus structure formation was evident in all treatments, except in the control treatment that did not include the presence of the growth regulator. As auxin concentration increased, callus formation increased until the greatest response was achieved with 2.0 mg L^{-1} Picloram (Table 1).

At the maximum concentration of Picloram, yellow embryogenic masses were observed (Figure 3AB), as well as somatic embryos in globular stage, which were easily separated since there was no vascular connection with the maternal tissue (Figure 3C).

These results differ from those obtained by other authors (16), where they state that the use of 2,4-D, ANA and Picloram (1.0 mg L^{-1}), induced the formation of callus in explants leaves and petioles; however, for the nodal segments of *D. alata* varieties 'Kinampay and 'VU-2' after six weeks in the dark behaved similarly, achieving 78 % callus formation, while in petioles and leaves the callus were of soft consistency and with more than 50 % of their area necrotic, due to the phenolic compounds exuded from the cut areas.

Table 1. Callus formation percentage with different concentrations of Picloram from yam nodal segments at 30 days of culture

Treatments	Picloram (mg L^{-1})	Nodal segmentss
1	0.0	0^{e}
2	0.5	28^{d}
3	1.0	50^{c}
4	1.5	65^{b}
5	2.0	80^{a}

Different letters indicate significant statistical differences according to the proportions comparison test (p≤0.05)

The results of the present investigation contrast with those previously published, where when using higher concentrations of Picloram 9.0 mg L^{-1}, results lower than 43 % were obtained in axillary buds (5). For this reason, it is suggested that in some plant species, it is required to combine Picloram with other auxins such as 2,4-D to induce the formation of embryogenic callus (17).

Picloram has been successfully used for the induction of friable embryogenic callus in root and tuber crops, especially cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*) and yam (12,16). In this sense, it was demonstrated that it is possible to induce pro-embryogenic masses in hawthorn yam, with a percentage higher than 90 %, from leaves with petiole under the effect of a concentration 2 mg L^{-1} of Picloram (18).

However, the results obtained in the present experiment demonstrate the essential role played by Picloram in callus formation, the best results being obtained in explants from nodal segments.

CONCLUSIONS

An 80 % of nodal segments with embryogenic callus formation, of yellowish-brown color, compact and friable consistency, were achieved with the use of 2.0 mg L^{-1} of Picloram in *Dioscorea rotundata* cultivar Blanco de Guinea.

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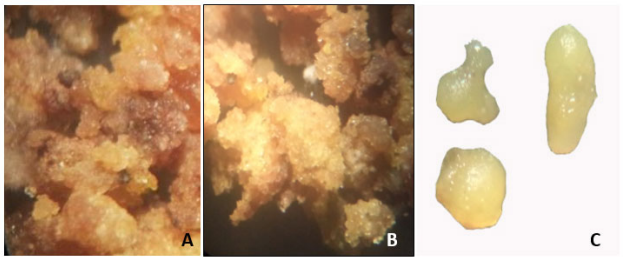

AB) Callus with somatic embryosmasses of yam. C) Somatic embryos at globular stage

Figure 3. Embryogenic callus of *Dioscorea cayenensis* subsp rotundata Poir 'Blanco de Guinea' in MS culture medium with Picloram 2.0 mg L^{-1} at 30 days of culture

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