Callogenesis in leaves of *Kalanchoe pinnata* Lam. by 2,4-D and BA action

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ABSTRACT: The *Kalanchoe pinnata* Lam. is a bush species of the Crassulaceae that is distinguished by its important medicinal properties. Its leaves are used as cataplasm to treat headaches and wounds. There is evidence for a hypotensive and anti-inflammatory effect. Techniques of plant tissue culture have been applied to plant species that produce substances likely to be explored in pharmacology, cell suspension being the main technique. At the industrial level, this method utilizes bioreactors in order to produce secondary metabolites on a large scale. The objective of this study was to evaluate the effects of *in vitro* combinations of 2,4-dichlorophenoxiacetic acid (2,4-D) and benzylaminopurine (BA) on callus induction in leaf explants of *K. pinnata*. Leaf fragments were inoculated in MS medium supplemented with 3.0% sucrose, 0.8% agar and factorial combinations of 2,4-D (0.00, 4.52, 9.06, 18.12 µM) and BA (0.00, 4.44, 8.88, 17.76 µM). The cultures were kept in the darkness at 24±2ºC for 50 days. The percentage of callus induction and the area of explants covered by callus cells were evaluated. In the absence of growth regulators, callus induction did not occur, with necrosis of all explants. The highest percentage of callus induction was 100%, obtained with the combination of 9.06 µM 2,4-D and 8.88 µM BA, but the calluses covered only 25% of the leaf area. The most efficient combination was 4.52 µM 2,4-D and 8.88 µM BA, resulting in 91% callus induction with 50 to 100% of the explants being covered by callus cells.

Keywords: Medicinal plant, growth regulators, cell suspension.

RESUMO: Calogênese em folhas de *Kalanchoe pinnata* Lam. pela ação de 2,4-D e BAP. *Kalanchoe pinnata* Lam. é uma espécie arbustiva da família Crassulaceae que apresenta interessantes propriedades medicinais. Suas folhas são utilizadas em cataplasma para tratar enxaqueca e ferimentos. Há evidência de seu efeito como hipotensiva e anti-inflamatória. Técnicas de cultura de tecidos vegetais têm sido aplicadas para espécies que possuem substâncias passíveis de exploração na farmacologia, sendo a suspensão celular a principal técnica utilizada. A nível industrial, este método utiliza biorreatores para produzir metabólitos secundários em larga escala. Este estudo teve como objetivo avaliar os efeitos *in vitro* de combinações do ácido 2,4-diclorofenoxiacético (2,4-D) e de benzilaminopurina (BAP) na indução de calos em explantes foliares de *K. pinnata*. Fragmentos foliares foram inoculados em meio MS contendo 3% de sacarose, 0,8% de ágar e combinações fatoriais de 2,4-D (0,00; 4,52; 9,06 e 18,12 µM) e BAP (0,00; 4,44; 8,88 e 17,76 µM). Os cultivos foram mantidos no escuro, a 24±2ºC por 50 dias. A porcentagem de indução de calos e a área dos explantes coberta por células de calos foram avaliadas. Na ausência de reguladores de crescimento não ocorreu indução de calos, com necrose de todos os explantes. A porcentagem mais alta de indução de calos foi de 100%, obtida com a combinação de 9,06 µM de 2,4-D e 8,88 µM de BAP, mas estes calos cobriram apenas 25% da área foliar. A combinação mais eficiente foi de 9,06 µM de 2,4-D e 8,88 µM de BAP, que resultou em 91% de indução e 50 a 100% da área dos explantes coberta por células de calos.

Palavras-chave: Planta medicinal, reguladores de crescimento, suspensão celular.

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INTRODUCTION

The increasing interest in natural products for medicinal use has led to difficulties in maintaining a constant supply of raw material while protecting the species from overexploitation. This question has stimulated the prospection and the establishment of *in vitro* plant cultures for production of secondary metabolites (Bertolucci et al., 2005). The *in vitro* techniques have been considered efficient methods for producing important substances for pharmacological exploration (Kerbauy, 1997). Among the techniques, the most widely used is cell suspension that allows large scale production of secondary metabolites in bioreactors (Cid, 1998). In addition, the compounds content is changed in these systems as an effect of the level of tissue differentiation. These peculiarities have encouraged the development of several studies to evaluate the production of compounds by calluses with different characteristics, mainly concerning consistence and morphogenic potential (Flores & Nicoloso, 2007). For this, the establishment of efficient protocols determining the induction and maintenance of friable calluses is needed (Kollávorá, 2004; Oksman-Caldentey & Inzé, 2004).

Kalanchoe pinnata Lam. is an African bush plant of the Crassulaceae botanical family largely spread over Saudi Arabia, Yemen, Central Africa, Madagascar, and tropical areas of Asia, Australia and South America (Khan et al., 2006). Its leaves are used as a cataplasm to treat headache and boils, by placing heated leaves over the affected area; and as a healing treatment for burns and wounds, applying a leaf paste on them. The leaf juice is obtained by blending it with water and it is drunk between the meals in the treatment of gastritis and ulcers. There is also evidence for its effect as a hypotensive, antirheumatic and anti-inflammatory agent (Almeida et al., 2000). The bufadienolides present in *K. pinnata* leaves have anti-tumor activity, highlighting bryophyllin for its strong activity. The presence of flavonoids contraindicates its use by people with hyperthyroidism because they are inhibitors of thyroperoxidase. The anti-cholinesterase activity is also related to this species which enhances the depressing response of acetylcholine, contraindicating its use by hypotensive people (Almeida *et al*. 2000).

The stage of friable callus is required for the establishment of cell suspension protocols. The objective of this work was to evaluate the effect of combinations of 2,4-dichlorophenoxiacetic acid (2,4-D) and benzylaminopurine (BA) on callus induction from leaf explants of *K*. *pinnata*.

MATERIAL AND METHOD

The experimentation was carried out at the Laboratory of Plant Tissue Culture of the Brazilian Agricultural Research Corporation (EMBRAPA) in Porto Velho, Brazil. Leaves of *K*. *pinnata* were collected at the experimental field and disinfected by washing them with sponge and a detergent agent in sterile distilled water for five minutes, followed by immersion in 70% (v/v) ethanol for one minute and 1% (w/v) calcium hypochlorite for 30 minutes and three rinses in sterile distilled water. The leaves were cut into 1.0 cm^2 fragments containing the midrib, which were inoculated individually in test tubes with half strength MS (Murashige & Skoog, 1962) salts and vitamins. The medium was supplemented with sucrose (3.0%), agar (0.8%), 2,4-D (0.00, 4.52, 9.06, 18.12 µM) and BA (0.00, 4.44, 8.88, 17.76 µM) in factorial combination (4 x 4) totaling 16 treatments.

The pH was adjusted to 5.8 before autoclaving (120ºC for 20 minutes). After inoculation the cultures were kept in darkness at 24±2ºC for 50 days. The experimental design used was entirely random, with 16 treatments and four replicates of five explants each. The percentage of callus induction (%CI) and the leaf area covered by callus cells (%LACC) were evaluated according to methodology described by Santos et al. (2013). Data were subjected to regression analysis.

RESULT AND DISCUSSION

Callus formation was not achieved in leaf segments of *K. pinnata* inoculated in the absence of growth regulators. However, shoot induction and subsequent plantlet formation have been observed. Callus induction started at 20 days of culture with swelling of the explants. At 50 days white and friable calluses were observed in all the treatments but in the experimental control. The concentrations of BAP and 2,4-D alone and in interaction affected significantly the variable percentage of callus induction in the explants. In the regression analysis the model that has been better adjusted to the 50 days data was the quadratic polynomial with all values of R^2 above 0.89.

As can be inferred by the observation of the results in Figure 1, the adequate hormonal balance would be among the combinations of concentrations from 4.52 to 9.06 µM 2,4-D and from 8.88 to 17.76 µM BA. In the absence of growth regulators, callogenesis was not observed, but the direct induction of shoots and subsequent formation of plantlets did occur. Lima et al. (2008) have also studied the interaction of different concentrations of 2,4-D with TDZ or BA and of NAA with BA on callus induction in leaf segments of *Croton urucurana*. They

observed that there was no callus formation in the absence of growth regulators.

The importance of 2,4-D is evidenced by observing that in the treatments without this regulator the callus induction did not occur (0.00, 8.88 or 17.76 µM BA) or it was very low (33.3% in the treatment with 4.44 µM BA). In the same way, it can be also observed that the presence of BA is important for induction, because in the treatments without this regulator callus induction did not occur (0.00, 9.06 or 18.12 µM 2,4-D), or occurred (4.52 µM 2,4-D) in 62.5% but with no great area covered by callus cells (zero to 50%), as can be observed in Table 1.

In general terms, the combination of the two growth regulators promoted the increase of the two variables - callus induction and area covered by callus cells. The higher percentage of callus induction was obtained with the combinations: 4.44 µM BA + 4.52 µM 2,4-D (87.5%); 8.88 µM BA + 4.52 µM 2,4-D (90.9%); 8.88 µM BA + 9.06 µM 2,4-D (100.0%); 17.76 µM BA + 4.52 µM 2,4-D (87.5%). Considering the area of the explants covered by callus cells, the combination of 8.88 µM BA + 4.52 µM 2,4-D was the most efficient, resulting in 27.3% of the explants with 51 to 75% covered by callus cells and 63.6% with 76 to 100%.

The growth regulator 2,4-D is the most often used auxin in callogenesis and has been referred to as essential in some cases. The auxins are able to start cell division and to control the processes of growth and cell elongation (Nogueira et al., 2007). In general, slightly similar concentrations of auxins and cytokinins in the culture medium promote callus induction, but the responses to interactions of these classes of growth regulators can vary according to the regulator, explant and genotype peculiarities (Cordeiro et al., 2007). They can act together in synergistic interaction or not, leading to dedifferentiation (Santos, 2001). These interactions have been used and tested in different forms to establish and to refine the exact concentrations in each situation (Grattapaglia & Machado, 1998).

Thomé et al. (2004) studied the micropropagation of *Kalanchoe blossfeldiana* Poelln. and observed 100% of callogenesis in leaf explants by using 4.44 µM BA + 0.16 µM NAA with subsequent organogenesis in two cultivars, Gold Trike and Klabat, using leaves and petioles fragments as explants. No direct organogenesis, but adventitious buds from calluses were observed in 100% of the explants with the supplementation of the MS medium with 0.225 µM BA + 0.006 µM NAA. Cerqueira (1999) also achieved high callus induction in leaf explants of *Tridax procumbens* Linn. with 0.372 µM NAA + 0.450 µM BA, observing 100% of the explants area covered by callus cells. Azevedo (2003) acquired calluses formation from *Copaifera langsdorffii* Defs. in MS medium with 0.372 µM 2,4-D + 0.225 µM BA, in the light. Zanotti et al. (2012) observed the highest frequencies of primary calluses in leaf explants of *Copaifera langsdorffii* utilizing MS medium supplemented with combinations of 15.0 µM BA and 5.0 µM 2,4-D.

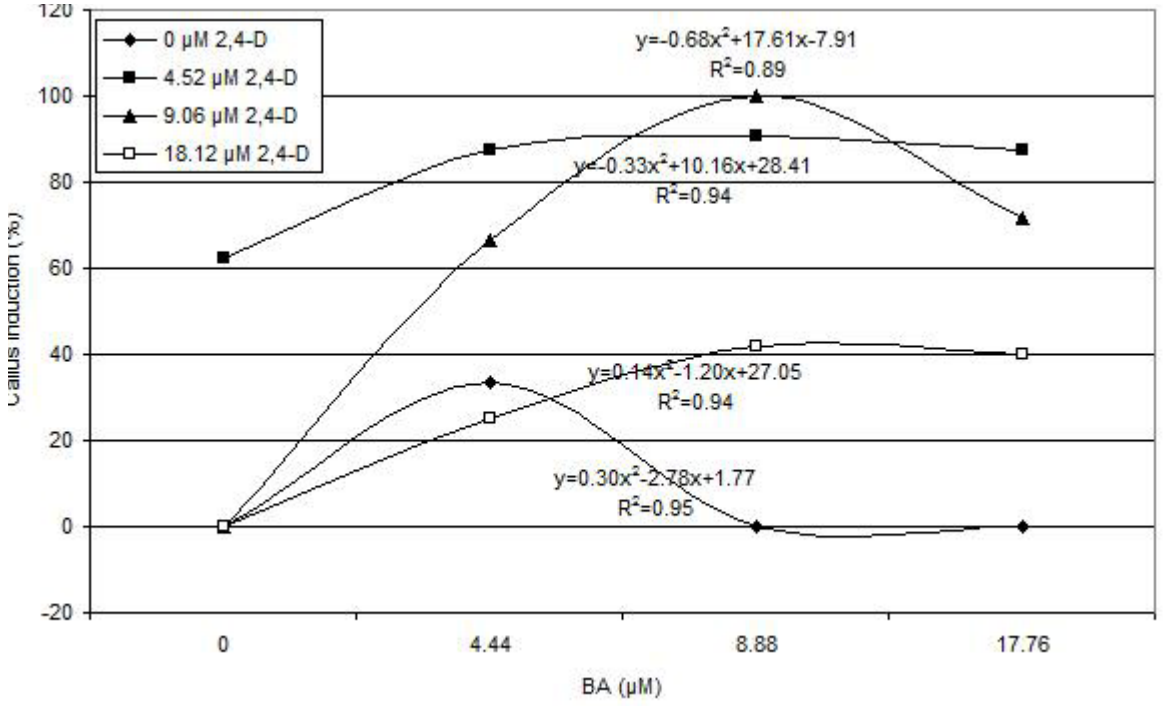

FIGURE 1. Effect of BA and 2,4-D on callus induction in *K. pinnata* leaf explants after 50 days of cultivation.

TABLE 1. Effect of combinations of BA and 2,4-D on callus induction in *K. pinnata* leaf explants after 50 days of cultivation.

Growth regulators (µM)		Total induction (%)	Percentage of the explants area covered by callus cells			
BA	2,4-D		0-25%	26-50%	51-75%	76-100%
-	-	0.0	0	0	0	0
-	4.52	62.5	50.0	12.5	0	0
-	9.06	0.0	0	0	0	0
-	18.12	0.0	0	0	0	0
4.44	-	33.3	33.3	0	0	0
4.44	4.52	87.5	0	25.0	37.5	25.0
4.44	9.06	66.7	0	33.3	33.3	0
4.44	18.12	25.0	0	0	25.0	0
8.88	-	0.0	0	0	0	0
8.88	4.52	90.9	0	0	27.3	63.6
8.88	9.06	100.0	100	0	0	0
8.88	18.12	41.7	25	16.7	0	0
17.76	-	0.0	0	0	0	0
17.76	4.52	87.5	12.5	25	12.5	37.5
17.76	9.06	71.4	0	7.1	42.9	21.4
17.76	18.12	40.0	0	0	40	0

Callus production is an important aspect in the medicinal plants *in vitro* culture allowing the synthesis of important compounds in higher amounts when compared with other methods of cultivation of *Kalanchoe pinnata*.

CONCLUSIONS

Callus induction in *K. pinnata* leaf explants needs growth regulators supplementation in the medium.

The interaction of BA and 2,4-D is positive in this concern.

The combination of 4.52 µM 2,4-D + 8.88 µM BA was the most efficient, resulting in 91% callus induction with 50 to 100% of the explants area covered by callus cells.

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