Searching an auxinic herbicide to use as positive control in toxicity assays

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Abstract: Due to rising concerns for environmental and human health, many toxic compounds, such as auxin-based herbicides, have been tested in relation to their toxicity effect. Especially cyto- and phytotoxic assays have been performed on a number of monocot and eudicot plant species. In these approaches the toxicity level of the auxin is compared to a positive control – usually a commercial compound with known effects and chemical similarity to the target compound. However, many target compounds still lack an indication of an adequate positive control. Here, we evaluate the phytotoxic and cytotoxic effect of the auxins 2,4-dichlorophenoxyacetic acid, dicamba, and picloram in order to test their potential use as positive controls. All tested auxinic herbicides showed clastogenic and aneugenic effect mechanisms. The results indicate 2,4-dichlorophenoxyacetic acid as the most phytotoxic and cytotoxic in the discontinuous method in *Lactuca sativa* L. and *Allium cepa* L., and also in the continuous method in *A. cepa*. Thus, we suggest 2,4-dichlorophenoxyacetic acid as a positive control for future mutagenesis studies involving new auxins. For studies with *L. sativa* in continuous method, we recommend the auxin picloram as positive control as this one was the only one which allowed the development of roots.

Key words: 2,4-D, cytotoxicity, dicamba, mutagenesis, phytotoxicity, picloram.

INTRODUCTION

The extensive and indiscriminate use of herbicides has provoked rising concerns about the environment and human health (Ribeiro & Lima 2011, Boccolini et al. 2013, Fagundes et al. 2015). On the other hand, the application of herbicides has contributed to evermore successful crop productivity (Song 2014, Ferreira 2015). It has thus become important to develop auxin herbicides which are as environment friendly as possible (Song 2014, Pinheiro et al. 2015, Alves et al. 2018). A common method to evaluate the toxicity effect of auxin herbicides are plant bioassays, such as cyto- and phytotoxicity assays (Aragão et al. 2015, Pinheiro et al. 2015). Phytotoxicity assays test the toxic effect of the target compound on germination and initial development of the seedlings, while cytotoxicity assays evaluate the damage in the cell cycle (mitotic index, nuclear alterations), in the chromatin (structural aberrations) and in the chromosome number (eu- and/or aneuploidy) (Andrade et al. 2008, 2010, Fernandes et al. 2009, Aragão et al. 2017, Costa et al. 2017, Silveira et al. 2017). These assays allow consequently to classify the target compound according to its effect mechanism as clastogenic and/or aneugenic (Vidaković-Cifrek et al. 2002).

Studies using such assays to evaluate the toxicity of auxin herbicides should compare the results with other compounds that promote
alterations at cytological and sporophytic levels, as a positive control (PC). These PC compounds should be of the same chemical class as the target compound and promote a large number of cytotoxic and phytotoxic alterations. Consequently, the PC is a fundamental parameter in these studies, providing information on the damage caused by the target compound (Fernandes et al. 2009).

A commonly used PC to evaluate the toxic effects of new herbicides is glyphosate. This common herbicide applied in agriculture worldwide (Powles & Yu 2010) has a well-studied effect mechanism (Pinheiro et al. 2015, Alves et al. 2018), which allows for a good comparison and understanding of the target molecule as well (Fernandes et al. 2009). However, glyphosate is not an auxin compound and only promotes an aneugenic effect (Powles & Yu 2010, Pinheiro et al. 2015), making it unsuitable for testing of auxin-based herbicides.

Two model plants normally used in these assays are the eudicot *Lactuca sativa* L. (2n=2x=18) and the monocot *Allium cepa* L. (2n=2x=16), because they have relatively few and big chromosomes facilitating microscopic evaluation (Andrade et al. 2010, Andrade-Vieira et al. 2014, Aragão et al. 2015). These species also have high sensitivity to the main PC compounds, and correlation with other groups, including mammals (Grant 1982, Fiskesjö 1985, Silveira et al. 2017). Also, *L. sativa* and *A. cepa* produce a high number of small seeds, have a fast initial development, as well as cheap and easy acquisition in specialized shops for agricultural products (Costa et al. 2017, Silveira et al. 2017).

Because of the need for an auxinic PC in mutagenesis approaches involving new auxin herbicides, the objective of this study was to verify the cyto- and phytotoxic effect of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), dicamba and picloram in order evaluate a potential use of these compounds as a PC.

**MATERIALS AND METHODS**

**Plant material**

Seeds of two species were used in the toxicity analyses:

a) *Lactuca sativa* (eudicot) ‘Crespa Grand Rapids’ (Isla Pak) – germination rate of 97%, 100% purity and within valid period,

b) *Allium cepa* (monocot) ‘Baia Periforme’ (Top Seeds) – germination rate of 80%, 99.6% purity and within valid period.

**Herbicides solutions**

The herbicides picloram (Sigma – Aldrich, ≥98%), dicamba (Sigma life Science, ≥98%) and 2,4-D (Sigma life Science, ≥98%) were used at 0.01% — the same concentration recommended for commercial glyphosate (N-(phosphonomethyl) glycine acid equivalent and inert ingredients), which we applied as positive control (PC). Distilled water was used as a negative control (NC).

For each of the eight treatments, we conducted five repetitions (Petri dishes) with twenty-five seeds each. Multiplied by the two species (*L. sativa* and *A. cepa*) and the two treatments (continuous and the discontinuous), resulting in a total of 160 petri dishes.

**Phytotoxicity assay**

In the continuous method, the seeds were germinated directly in the eight treatments and NC. After, we measured the following parameters for *L. sativa*: germinated seed percentage (GP) after 48h, germination speed index (GSI) calculated every 8h during 48h, growth root (GR) and aerial growth (AG) measured after 48h and after 120h, respectively (Aragão et al. 2015, Pinheiro et al. 2015).
Except for AG (Silveira et al. 2017), the same parameters were verified for *A. cepa* partially at different time intervals: GP and GR were evaluated after 96h, and the GSI every 12h, during 96h (Bernardes et al. 2015, Silveira et al. 2017).

For the discontinuous method, the seeds were previously germinated in distilled water. Roots with a length of 1-2cm were exposed to the treatments for 24h (Aragão et al. 2015, Pinheiro et al. 2015, Silveira et al. 2017). Different from the continuous method, the RL and the AG parameters in *L. sativa* in the discontinuous method were evaluated after 24 and 72h, respectively. The parameter AG was not evaluated for *A. cepa*.

**Citotoxicity assay**

The citotoxicity assay followed the same experimental design of the phytotoxicity assay, using *A. cepa* and *L. sativa* as a reference. For this, ten roots from each Petri dish were collected after 96h for *A. cepa* and 48h for *L. sativa* (Silveira et al. 2017) in the treatments. The roots were placed in methanol: acetic acid (3:1/vv), with two changes: one after 10min, the second after 24h. Then the roots were stored at -20°C for at least 24h.

The roots were washed three times for 10 min each in distilled water and hydrolyzed in 5N HCl at 25°C for 18 min. Two root meristems were cut, placed on slides and stained with 2% acetic orcein. Five slides were prepared for each treatment and species.

The slides were analyzed under a light microscope (Nikon) with a 40-times magnification objective. Images were captured with an Eclipse 80i microscope (Nikon), using 100-times magnification. One thousand cells were evaluated in each slide, totaling to five thousand cells for each treatment for each plant species. Mitotic index (MI – number of mitosis cells/ number of cells), chromosome (CA – total of chromosome alterations cells/ number of cells) and nucleus alterations (NA – total of nuclear alterations cells/ number of cells) were identified and calculated (Fiskesjö 1985).

NA and CA were discriminated, categorized and frequencies for each were measured:

a) for NA – condensed nucleus, micronucleus (number of each NA/ number of cells),

b) for CA – C-metaphasis, chromosome stick, chromosome bridge, chromosome laggard, chromosomal fragment (number of each CA/ number of mitosis cells) (Pinheiro et al. 2015).

Besides, the frequency of each CA and total CA was calculated for each mitosis phase.

**Statistical analysis**

Variance analysis was performed in order evaluate the treatments. Means were compared with a Dunnett test (*p*<0.05), since this test is the most indicative to compare the treatments with the control (McHugh 2011). All analyses were carried out using the statistic program Genes (Cruz 2013).

**RESULTS AND DISCUSSION**

Our results show that between the negative control (NC), glyphosate and the auxin herbicides, glyphosate was the most cytotoxic (aneugenic) and phytotoxic, showing most similarity with the auxinic compounds (Figure 1). The auxins showed cytotoxicity and phytotoxicity (Figure 2), but varying in rate and with type of alteration for each method (continuous and discontinuous) and plant species (*A. cepa* and *L. sativa*).

Based on the cyto- and phytotoxicity assays, the 2,4-D was most similar to glyphosate in terms of the cito- and phytotoxic impact on the two species (except for the continuous method with *L. sativa*, Figures 1 and 2). The seeds of *L.
The tested auxinic herbicides showed different rates and types of toxicity. This result can be due to their chemical classes, which are divided into subgroups according to the molecular formula: 2,4-D belongs to the subgroup of the phenoxy-carboxylic acids, dicamba to benzoic acids, and picloram to pyridineacids. These molecule structure differences can result in distinct molecular interactions with the cells of \( L. \) \textit{sativa} (eudicot) and \( A. \) \textit{cepa} (monocot), influencing the toxic effect of the compounds on these model organisms (Christoffolet et al. 2015).

The cyto- and phytotoxic effect of 2,4-D was more similar to glyphosate than to the other auxinic herbicides (Figures 1 and 2). This result indicates a higher potential of 2,4-D as a PC than picloram or dicamba. Based on this, we recommend 2,4-D as a PC for other studies that evaluate the toxicity of new auxin compounds. One of the effects of 2,4-D is the slowing of
Figure 2. Parameters of cytotoxicity of auxins, in *Lactuca sativa* (a and b) discontinuous method, (c and d) continuous method and *Allium cepa* (e and f) discontinuous method, (g and h) continuous method. Means followed by (a) are statistically similar (Dunnett’s *p*<0.05) from negative control (water). Means followed by (b) are statistically similar (Dunnett’s *p*<0.05) from positive control (glyphosate). Abbreviations: GP – germinated seed percentage; GSI – germination speed index; GR – growth root; AG – aerial growth.
the cytoplasmatic flow due to its capacity to remove the actin filaments in the cytoskeleton (Rahman et al. 2007), which alters the mobility of the organelles (Rodríguez-Serrano et al. 2014). This systematic effect of cellular alteration probably caused the metabolic dysfunction, since the normal metabolism of the peroxisome, chloroplasts and mitochondria depend on the sharing of metabolites (Rodríguez-Serrano et al. 2014). Similarly, glyphosate is also a systemic herbicide, but with an inhibitory effect on the aromatic amino acid production (Kirkwood & McKay 1994). Both, 2,4-D and glyphosate increase the ethylene production, promoting cell death (Grossmann 2007, Song 2014).

The root growth of _L. sativa_ in the continuous method only occurred in the picloram treatment (Figure 1b). Therefore, picloram is indicated as a PC to investigate the cytotoxicity of new auxin compounds from a system involving _L. sativa_ and continuous methods.

Dicamba was the least phytotoxic (GR and GSI) and cytotoxic (MI) (Figures 1 and 2). GR and GSI values were higher in dicamba than in glyphosate and the other auxins, showing a lower phytotoxic effect and better plant development (Aragão et al. 2017). Corroborating these results, we also observed less cytotoxicity of dicamba in the MI data, indicating a small effect of the auxin on cell cycle, plant growth and development.

GR was inhibited in all methods and in both species, while GP and GSI decreased only in _L. sativa_ (Figure 1). These results can be related to the sensitivity of the parameters in the evaluated toxicity: GP is considered to be less sensitive (Aragão et al. 2017). GSI showed a delay in germination and a decrease in plant development (Costa et al. 2017), while GR was the most sensitive parameter evaluated in the phytotoxicity assay (Valério et al. 2007).

All tested auxins promoted the CAs: chromosomal stick, multipolar, chromosomal delay and c-metaphasis (Figures 2 and 3). Besides, _A. cepa_ also increased in the number of chromosomal breaks and bridges (Figure 2f, h).

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Figure 3. Examples of chromosomal alterations in meristematic cells of roots tips from _L. sativa_ after exposure to 2,4-D, picloram and dicamba. a and b) anaphase bridge; c) chromosome adherence; d and e) lost chromosomes; f) c-metaphasis. Bar = 10 µm.
Our determination of the effect mechanisms of the molecules in mitosis through CA (Bernardes et al. 2015) showed that the tested auxins are clastogenic and aneugenic compounds. Aneugenicity, as found in A. cepa and L. sativa, is characterized by the destabilization or the malfunction of the mitotic spindle (Fernandes et al. 2009, Andrade-Vieira et al. 2011, 2014, Bernardes et al. 2015). All three auxins can additionally be classified as clastogenic compounds due to the increase of chromosomal break and chromosomal bridge found in all treatments in A. cepa (Figure 2f, h). These clastogenic changes occur when the toxic molecule affects the chromatin directly or indirectly, resulting in culminating chromosomal damage (Fernandes et al. 2009, Andrade-Vieira et al. 2011, 2014, Bernardes et al. 2015).

The different types of CA observed in A. cepa and L. sativa (Figure 2b, d, f and h) can be related directly to the model organisms, but the data found for these species were complementary, indicating the need to further evaluate these and other models to obtain better knowledge on the compounds. But according to Silveira et al. (2017), A. cepa is more sensitive to cytotoxicity parameters than L. sativa.

In conclusion, our results show that 2,4-D is the most cyto- and phytotoxic auxin tested in this study, which is why we suggests its use as an auxinic herbicide as PC in future phytotoxic and cytotoxic assays and mutagenesis studies. Only the specific system involving L. sativa in continuous method, indicating picloram as the best PC. All auxinic herbicides showed clastogenic and aneugenic effect mechanisms. L. sativa was more sensitive to the phytotoxic parameters, while A. cepa was more sensitive to cytotoxic parameters.

Acknowledgments
We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Federação de Amparo à Pesquisa Espírito Santo (FAPES/UNIVERSAL, Vitória-ES, Brazil; Grant 180/2017 and 80707114/18). This study was partly funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001.

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How to cite

ALVES TA, ROBERTO CEO, PINHEIRO PF, ALVES TA, HENRIQUE MKC, FERREIRA A, CLARINDO WR & PRAÇA-FONTES MM. 2021. Searching an auxinic herbicide to use as positive control in toxicity assays. An Acad Bras Cienc 93: e20181262. DOI 10.1590/0001-376520120181262.

Manuscript received on November 28, 2018; accepted for publication on October 21, 2019

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