An Acad Bras Cienc (2021) 93(1): e20181262 DOI 10.1590/0001-3765202120181262
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CROP SCIENCE

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

THAMMYRES A. ALVES, CARLOS EDUARDO O. ROBERTO, PATRÍCIA F. PINHEIRO, THAYLLON A. ALVES, MAIKON K.C. HENRIQUE, ADÉSIO FERREIRA, WELLINGTON R. CLARINDO & MILENE M. PRAÇA-FONTES

Abstract: Due to rising concerns for environmental and human health, many toxic compounds, such as auxin-based herbicides, have been tested in relation their toxicity effect. Especially cyto- and phytotoxic assays have been performed on a number 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 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 phyto- 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 ($3:1/vv^{-1}$), with two changes: one after 10min, the second after 24h. Then the roots were stored at -20ºC for at least 24h.

The roots were washed three times for 10 min each in distilled water and hydrolyzed in 5N HCl at 25ºC 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.*

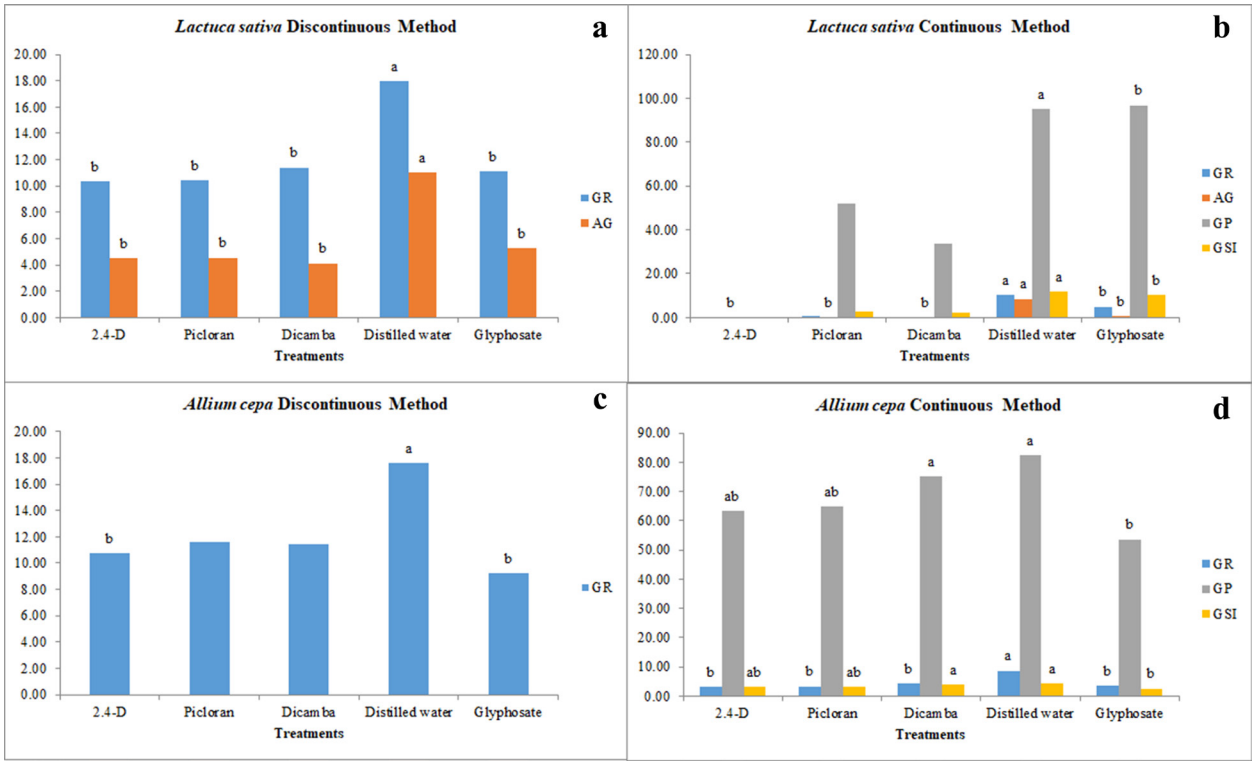

Figure 1. Parameters of phytotoxicity of auxins, in *Lactuca sativa* (a) discontinuous method, (b) continuous method and *Allium cepa* (c) discontinuous method, (d) continuous method. Means followed by (a) are statistically similar (Dunnett's $p<0.05$) to positive control (glyphosate). Abbreviations: MI – mitotic index; CA – chromosome alterations; NA – nucleus alterations; MNC – micronucleus; NC – nucleus condensed; lost – chromosome laggard; multipolar – multipolarity; stick – chromosome stick; C-metaphasis – chromosomes in C-metaphasis; bridge – chromosome bridge, delay – chromosome with delay; break – chromosomal fragment.

sativa in the continuous method did not root when exposed to auxins other than picloram, impeding the cytotoxicity assay. Thus, picloram was the best treatment under this condition (Figures 1b and 2d). Dicamba was the auxin least similar in effect to glyphosate, showing greater GP, GSI and MI than glyphosate, 2,4-D and picloram (Figures 1 and 2).

All auxins interfered in the GR of the two species. However, the germination parameters (GP and GSI) only decreased in *L. sativa*, indicating that the phytotoxic effect of the auxins was higher than that of glyphosate (Figure 2).

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. sativa* (eudicot) and *A. 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

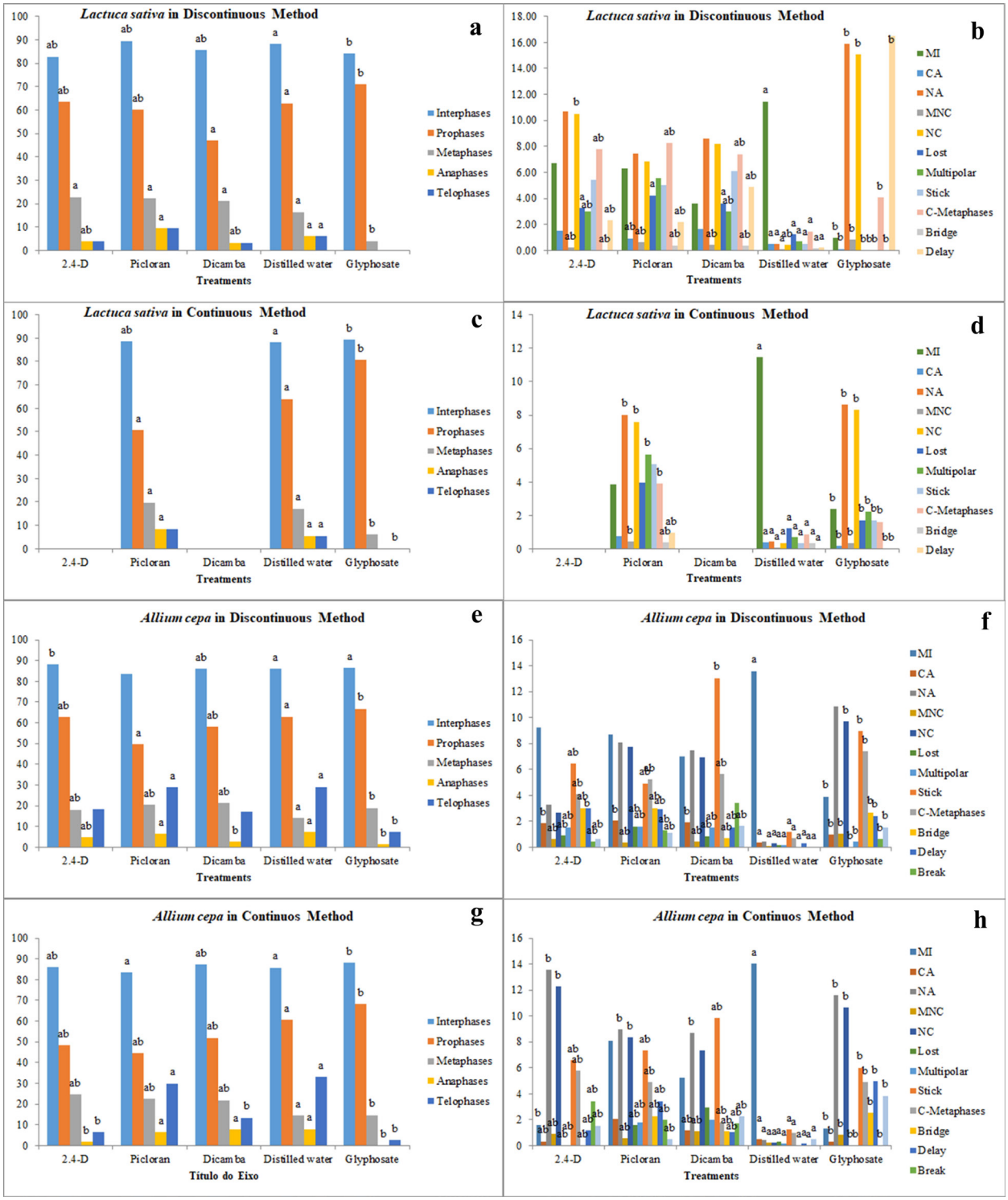

Figure 2. Parameters of cytotocixity of auxins, in *Lactuca sativa* (a and b) discontinuous method, (c and d) continuous method and *Allium cepa* (e and f) discontinuous method, (g and h) continuous method. Means followed by (a) are statistically similar (Dunnett's $p<0.05$) from negative control (water). 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)

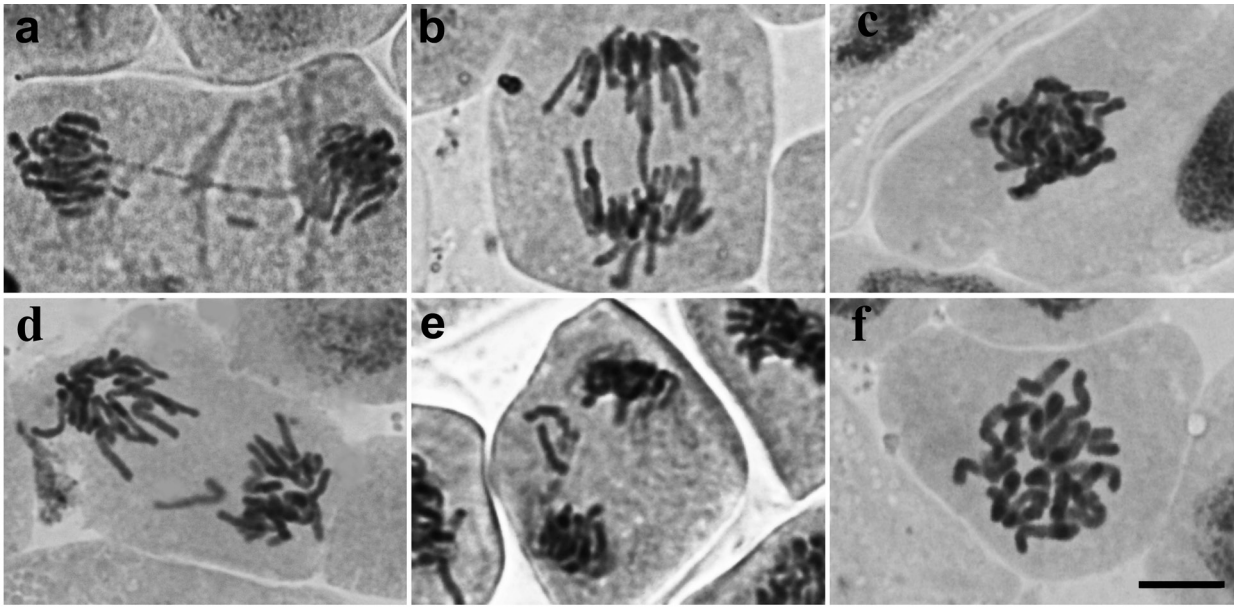

Figure 3. Examples of chromosomal alterations in meristematic cells of roots tips from *L. sativa* after exposure to 2,4-D, picloram and dicamba. a and b) anaphase bridge; c) chromosome adherence; d and e) lost chromosomes; f) c-metaphasis. Bar = 10 µm.

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 phyto- 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.

REFERENCES

ALVES TA, PINHEIRO PF, PRAÇA-FONTES MM, ANDRADE-VIEIRA LF, CORRÊA KB, ALVES TA, DA CRUZ FA, LACERDA-JUNIOR V, FERREIRA A & SOARES TCB. 2018. Toxicity of thymol, carvacrol and their respective phenoxyacetic acids in *Lactuca sativa* and *Sorghum bicolor*. Ind Crops Prod 114: 59-67.

ANDRADE LF, CAMPOS JMS & DAVIDE LC. 2008. Cytogenetic alterations induced by SPL (spent potliners) in meristematic cells of plant bioassays. Ecotoxicol Environ Saf 71: 706-710.

ANDRADE LF, DAVIDE LC & GEDRAITE LS. 2010. The effect of cyanide compounds, fluorides and inorganic oxides present in spent pot liner on germination and root tip cells of *Lactuca sativa*. Ecotoxicol Environ Saf 25: 626-631.

ANDRADE-VIEIRA LF, BOTELHO CM, PALMIERI MJ, LAVIOLA BG & PRAÇA-FONTES MM. 2014. Effects of *Jatropha curcas* oil in *Lactuca sativa* root tip bioassays. An Acad Bras Cienc 86: 373-382.

ANDRADE-VIEIRA LF, GEDRAITE LS, CAMPOS JMS & DAVIDE LC. 2011. Spent Pot Liners (SPL) induced DNA damage and nuclear alterations in root tip cells of *Allium cepa* as a consequence of programmed cell death. Ecotoxicol Environ Saf 74: 882-888.

ARAGÃO FB, PALMIERI MJ, FERREIRA A, COSTA AV, QUEIROZ VT, PINHEIRO PF & ANDRADE-VIEIRA LF. 2015. Phytotoxic and cytotoxic effects of *Eucalyptus* essential oil on lettuce (*Lactuca sativa* L.). Allelopathy J 35: 259-272.

ARAGÃO FB, QUEIROZ VT, FERREIRA A, COSTA AV, PINHEIRO PF, CARRIJO TT & ANDRADE-VIEIRA LF. 2017. Phytotoxicity and cytotoxicity of *Lepidaploa rufogrisea* (Asteraceae) extracts in the plant model *Lactuca sativa* (Asteraceae). Rev Biol Trop 65: 435-443.

BERNARDES PM, ANDRADE-VIEIRA LF, ARAGÃO FB, FERREIRA A & DA SILVA-FERREIRA MF. 2015. Toxicity of difenoconazole and tebuconazole in *Allium cepa*. Water Air Soil Pollut 226: 207.

BOCCOLINI PM, BOCCOLINI CS, CHRISMAN JR, MARKOWITZ SB, KOIFMAN S, KOIFMAN RJ & MEYER A. 2013. Pesticide use and non-Hodgkin's lymphoma mortality in Brazil. Int J Hyg Environ Health 216: 461-466.

COSTA AV ET AL. 2017. Synthesis of Novel Glycerol-Derived 1,2,3-Triazoles and Evaluation of Their Fungicide, Phytotoxic and Cytotoxic Activities. Molecules 22: 1666-1681.

CRUZ CD. 2013. GENES – a software package for analysis in experimental statistics and quantitative genetics. Acta Sci Agron 35: 271-276.

FAGUNDES MZ, GONÇALVES MA, SOARES MP, MARTINS ML, ZANELLA R, RIET-CORREA F & ANJOS BL. 2015. Clinico pathological and toxicological aspects of poisoning by the clomazone herbicide in sheep. Small Rumin Res 124: 120-126.

FERNANDES TCC, MAZZEO DEC & MARIN-MORALES MA. 2009. Origin of nuclear and chromosomal alterations derived from the action of an aneugenic agent—Trifluralin herbicide. Ecotoxicol Environ Saf 72: 1680-1686.

FERREIRA MLPCA. 2015. Pulverização aérea de agrotóxicos no Brasil: cenário atual e desafios. R Dir Sani 15: 18-45.

FISKESJÖ G. 1985. The *Allium* test as a standard in environmental monitoring. Hereditas 102: 99-112.

GRANT WF. 1982. Cytogenetic studies of agricultural chemicals in plants. In: Fleck RA and Hollaender A (Eds), Genetic toxicology: an agricultural perspective. New York: Plenum Press, p. 353-378.

GROSSMANN K. 2007. Auxin herbicide action: lifting the veil step by step. Plant Signal Behav 2: 421-425.

KIRKWOOD RC & MCKAY I. 1994. Accumulation and elimination of herbicides in select crop and weed species. J Pest Sci 42: 241-248.

PINHEIRO PF, COSTA AV, ALVES TA, GALTER IN, PINHEIRO CA, PEREIRA AF, OLIVEIRA CM & FONTES MMP. 2015. Phytotoxicity and cytotoxicity of essential oil from leaves of *Plectranthus amboinicus*, carvacrol, and thymol in plant bioassays. J Agric Food Chem 63: 8981-8990.

POWLES SB & YU Q. 2010. Evolution in Action: Plants Resistant to Herbicides. Annu Rev Plant Biol 61: 17-47.

RAHMAN A, BANNIGAN A, SULAMAN W, PECHTER P, BLANCAFLOR EB & BASKIN TI. 2007. Auxin, actin and growth of the *Arabidopsis thaliana* primary root. Plant J 50: 514-528.

RIBEIRO JPN & LIMA MIS. 2011. Potencial alelopático de *Crinumamericanum* L. sob diferentes condições de extração. Semina: Ciênc Agrár 32: 465-472.

RODRÍGUEZ-SERRANO M, PAZMIÑO DM, SPARKES I, ROCHETTI A, HAWES C, ROMERO-PUERTAS MC & SANDALIO LM. 2014. 2,4-Dichlorophenoxyacetic acid promotes S-nitrosylation and oxidation of actin affecting cytoskeleton and peroxisomal dynamics. J Exp Bot 237: 1-11.

SILVEIRA GL, LIMA MGF, DOS REIS GB, PALMIERI MJ & ANDRADE-VIEIRA LF. 2017. Toxic effects of environmental pollutants: Comparative investigation using *Allium cepa* L. and *Lactuca sativa* L. Chemosphere 178: 359-367.

SONG Y. 2014. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. J Integr Plant Biol 56: 106-113.

VALÉRIO ME, GARCIA JF & PEINADO FM. 2007. Determination of phytotoxicity of soluble elements in soils, based on a bioassay with lettuce (*Lactuca sativa* L.). Sci Total Environ 378: 63-66.

VIDAKOVIĆ-CIFREK Z, PAVLIC, M, REGULA I & PAPES D. 2002. Cytogenetic damage in shallot (*Allium cepa*) root meristems induced by oil industry "high-density brines". Arch Environ Contam Toxicol 43: 284-291.

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 10.1590/0001-3765202120181262.

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

THAMMYRES A. ALVES[1]
https://orcid.org/0000-0003-1148-4832

CARLOS EDUARDO O. ROBERTO[2]
https://orcid.org/0000-0002-6210-7589

PATRÍCIA F. PINHEIRO[3]
https://orcid.org/0000-0003-4019-5773

THAYLLON A. ALVES[1]
https://orcid.org/0000-0003-2478-851X

MAIKON K.C. HENRIQUE[1]
https://orcid.org/0000-0001-6303-2729

ADÉSIO FERREIRA[4]
https://orcid.org/0000-0002-7000-1725

WELLINGTON R. CLARINDO[1]
https://orcid.org/0000-0002-8826-8620

MILENE M. PRAÇA-FONTES[1]
https://orcid.org/0000-0001-7738-9518

[1]Universidade Federal do Espírito Santo, Centro de Ciências Exatas, Naturais e da Saúde, Departamento de Biologia, Laboratório de Citogenética e Cultura de Tecidos, Alto Universitário, s/n, Caixa Postal 16, 29500-000 Alegre, ES, Brazil

[2]Universidade Federal do Espírito Santo, Centro de Ciências Exatas, Naturais e da Saúde, Departamento de Farmácia e Nutrição, Laboratório de Bioquímica e Biologia Molecular, Alto Universitário, s/n, Caixa Postal 16, 29500-000 Alegre, ES, Brazil

[3]Universidade Federal do Espírito Santo, Centro de Ciências Exatas, Naturais e da Saúde, Departamento de Física e Química, Laboratório de Química, Alto Universitário, s/n, Caixa Postal 16, 29500-000 Alegre, ES, Brazil

[4]Universidade Federal do Espírito Santo, Centro de Ciências Exatas, Naturais e da Saúde, Departamento de Física e Química, Laboratório de Biometria, Alto Universitário, s/n, Caixa Postal 16, 29500-000 Alegre, ES, Brazil

Correspondence to: **Milene Miranda Praça-Fontes**
E-mail: milenemiranda@yahoo.com.br

Author contributions

Thammyres de Assis Alves: paper conception, literature review, data collection, analyses, interpretation of results, manuscript writing; Carlos Eduardo de Oliveira Roberto and Maikon Keoma da Cunha Henrique: data collection and processing; Patrícia Fontes Pinheiro: data analysis and discussion of results; Thayllon de Assis Alves: data collection and processing, preparation of figures; Adésio Ferreira: statistical analisys and interpretation of results; Wellington Ronildo Clarindo: discussion of results, paper writing; Milene Miranda Praça-Fontes: paper conception, advisor professor, discussion of results, paper writing.