Germination, cytotoxicity and mutagenicity in Lactuca sativa L. and Passiflora alata Curtis in response to sewage sludge application

Allan Rocha Freitas
Faculdade do Futuro
Nathália Aparecida Bragança Fávaris
Universidade Federal do Espírito Santo
Rodrigo Sobreira Alexandre
Universidade Federal do Espírito Santo
Tatiana Silva Souza
Universidade Federal do Espírito Santo
Iasmini Nicoli Galter
Universidade Federal do Espírito Santo
Julcinara Oliveira Baptista
Universidade Federal do Espírito Santo
Paula Aparecida Muniz Lima
Universidade Federal do Espírito Santo
Tamyris Mello (tamyrisdemello@gmail.com)
Universidade Federal do Espírito Santo
Wagner Campos Otoni
Universidade Federal de Viçosa/UFV
José Carlos Lopes
Universidade Federal do Espírito Santo

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Abstract

The physical and chemical characteristics of the soil can influence plant growth. When sewage sludge (SS) is applied as a soil fertilizer, the accumulation of non-essential elements contained in it can be toxic for plants. The aim of this study was to understand the effect of SS dosage on the cell cycle of *Lactuca sativa* L. meristematic cells and on the initial growth of *L. sativa* and *Passiflora alata* Curtis. Nine concentrations of SS + distilled water (mg dm^{-3}) corresponding to 0, 20, 40, 60, 80, 120, 160, 320, and 520 t ha^{-1} were tested in four replicates of 25 seeds. Chemical analysis showed an increase in pH of the sludge from 0 to 80 t ha^{-1} SS followed by its stabilization thereafter. The highest electrical conductivity was observed at 520 t ha^{-1} SS. SS negatively affected the germination and initial growth of seedlings from *P. alata* and *L. sativa.* Cytogenetic analysis on 6000 *L. sativa* meristematic cells for each treatment revealed that SS could adversely affect the genetic stability of this species.

1. Introduction

Sewage sludge (SS) is a solid residue obtained from the treatment of domestic or industrial sewage. It is characterized by elevated organic matter content and a varied chemical composition (Kelessidis and Stasinakis 2012). To diminish the impact of excess waste residue, various practices for its utilization, such as industrial recycling, incineration, and especially its use as a plant fertilizer (Kelessidis and Stasinakis 2012; Grotto et al. 2013; Freitas et al. 2015), have been attempted in recent years.

Despite the benefits of applying SS to improve the physical and chemical characteristics of the soil and, therefore, plant growth (Freitas et al. 2015), the accumulation of certain sludge elements could be potentially toxic for plants (Singh and Agrawal 2008; Nascimento et al. 2014). The increased proportion of SS and the presence of shallower soils can amplify the concentration of toxic metals (Chu and He 2021), such as Cd, Ni, and Cu, whose increased bioavailability may explain changes in the soil microbial community (Markowicz et al. 2021). Moreover, the same authors reported that plasmids isolated from SS conferred β-lactam resistance or extreme resistance to tetracycline (> 256 μg mL^{-1}) via two antibiotic resistance genes (*bla* $_{NPS-2}$ and *tetA*) detected in SS-treated soil six months after fertilization. Accordingly, the application of SS to the soil, even at a low controlled dosage, can affect the soil microbiome and its resistance phenotype, which may have implications on public health.

Heavy metals may limit plant growth and development by inhibiting physiological processes, such as transpiration, respiration, and photosynthesis (Nagajyoti et al. 2010; Taiz et al. 2017). To prevent this from happening, it is necessary to know the chemical characteristics of the sludge and to determine the effect of different concentrations of SS on soil and plant performance. Santos et al. (2021) observed that using SS at concentrations of 50% or higher affected stomatal conductance, leaf temperature, and chlorophyll *a* fluorescence of *Alibertia edulis* (Rubiaceae) seedlings. This resulted in lower biomass, impaired growth, loss of leaf symmetry, and leaf deformations, which were possibly related to contamination with heavy metals. However, according to Chow and Pan (2020), pH and thermal treatment of this SS successfully removed any heavy metals from the pure biosolid, which was now safe

for use as fertilizer. Tests that quantify the impact of industrial waste and contaminated waters on plant morphological and cytogenetic parameters, such as phytotoxicity, cytotoxicity, genotoxicity, and mutagenic potential of chemicals, are routinely performed in laboratories (Felicidade et al. 2014). Alterations in the cell cycle have been associated with DNA damage elicited by environmental toxicity (Hoshina and Marin-Morales 2009; Singh et al. 2009).

Passiflora alata Curtis (sweet passion fruit) is increasingly popular in Brazil. The crop grows well when organic sources, such as SS, are added at a concentration of 80 t ha^{-1} (Freitas et al. 2015); however, it is unclear if the accumulated toxicity from SS components interferes with plant growth.

The use of plants as bio-indicators of cytotoxicity was pioneered in the 1970's (Grant 1999). *Lactuca sativa* L. has been widely studied to determine the phytotoxic, cytotoxic, and genotoxic effects of chemical compounds on plants, cells, and DNA, respectively (Andrade et al. 2010; Sousa et al. 2010).

The aim of the present study was to evaluate the effect of SS on the germination and initial growth of *P. alata* and *L. sativa*, as well as the effect on the cell cycle in meristematic cells of *L. sativa*.

2. Materials And Methods

The study was conducted at the Seed Analysis Laboratory of the Agricultural Sciences Center, Federal University of Espírito Santo, Alegre, ES, Brazil.

SS was collected according to the Brazilian Association of Technical Standards NBR 10007 (ABNT 2004) at the wastewater treatment plant belonging to the Espírito Santo State Company (Vitória, ES). The sludge was analyzed by the Agrolab - Analysis and Quality Control Company (Vila Velha, ES) to determine its physical and chemical characteristics, as well as concentration of As, Ba, Cd, Ca, Pb, Cu, Cr, S, Mg, Mn, Hg, Mo, N, Ni, K, Se, Na, S, Zn, P, and organic carbon and organic matter content (Table 1). Fractionation, extraction, and purification of organic carbon from the sludge was conducted in accordance with the International Humic Substances Society (IHSS 2016) as described by Thurman and Malcolm (1981) in analytical duplicate.

First, 4 g of sample was treated with 2 M H_3PO_4 in a 40-mL solution for 4 h in centrifuge tubes to extract free fulvic acids and light organic matter fractions. After stirring, the sample was centrifuged (HERMLE-Z382K centrifuge) at 5000 × *g* for 30 min. To extract alkali-soluble organic substances, the precipitate obtained in the previous step was adjusted to pH 7 and then 40 mL of 0.5 M NaOH was added to the centrifuge tube. The mixture (pH ~ 13) was shaken horizontally for 24 h to extract humic substances. The extract was centrifuged and the procedure was repeated three more times until complete extraction of humic substances was ascertained by their absence in the supernatant. Then, the organic carbon content in the supernatant containing humic substances and the residual precipitate, named humin fraction, was determined.

An aliquot of the humic substances extract was acidified to pH 1.5 with H_2SO_4 and then centrifuged to separate fulvic acids (supernatant) from humic acid (precipitate). Carbon content in each fraction and total organic carbon in the sludge samples was determined using acid dichromatometry, with ammonium ferrous sulfate as titrant and diphenylamine as an indicator.

Nine treatments corresponding to different SS concentrations of sludge + distilled water (mg dm^{-3}) at 20, 40, 60, 80, 120, 160, 320, and 520 t ha^{-1} plus distilled water (negative control) were assessed. The different proportions of SS served to determine the dosages that could benefit or inhibit *P. alata* (Freitas 2015). SS was sieved through a 2-mm mesh, and the solutions were prepared by shaking with the aid of a Marconi automatic mixer (model MA376/176) at 200 rpm and 25°C in two steps of 60 min each separated by a 30-min interval. After 24 h, the solutions (supernatants) were collected, pH and electrical conductivity (DM Digimed 31) were analyzed, and the solutions were stored in a refrigerator at 3 ± 1°C for further experiments.

The first experiment followed a completely randomized design with four replicates of 25 *L. sativa* seeds per treatment. The seeds were placed in Petri dishes with two sheets of Germitest® paper moistened with 5 mL of SS solution. Subsequently, the plates were kept in a biochemical oxygen demand incubator at a constant temperature of 24°C for 48 h in the dark (Brasil 2009).

The following variables were assessed: germination (%) and germination speed index (GSI). The latter was calculated according to Maguire (1962) as GSI = G1/D1 + ⋯ + Gn/Dn, where G1 = number of germinated seeds on the first day after sowing, Gn = number of germinated seeds 'n' days after sowing, D1 = one day after sowing, and Dn = 'n' days after sowing. Root growth (cm) was measured using a digital caliper (precision, 0.01 mm).

To perform the cytogenetic analysis of SS concentrations, seedling roots were collected and fixed in ethanol:acetic acid (3:1). Slides were prepared by crushing and stained with 2% acetic orcein. In each treatment, 6000 meristematic cells were analyzed and the following parameters were determined: number of prophase, interphase, metaphase, anaphase, and telophase cells; number of condensed nuclei, micronuclei, c-metaphase events, sticky chromosomes, bridges, and broken chromosomes; mitotic index [number of dividing cells (prophase, metaphase, anaphase, and telophase) / total number of analyzed cells × 100]; aberration index (number of cells with chromosomal aberrations / total number of cells analyzed × 100); and meristematic cell mutagenicity index (number of cells with micronuclei and breakage / total number of cells analyzed × 100).

In the second experiment, *P. alata* seeds from fruits harvested in São Mateus, in the north of Espírito Santo State were used. The seeds were manually extracted and processed with lime (added at 20% relative to the seed volume) for sarcotesta removal, washed in water, and laid out on Germitest® paper at 25°C for 24 h. Subsequently, the seeds were scarified with sandpaper (n° 100) and immersed in 500 mg L^{-1} gibberellic acid for 24 h.

Next, the seeds were placed in Petri dishes lined with two Germitest® sheets moistened with 5 mL SS solution for each treatment, coated with transparent plastic, and maintained in a biochemical oxygen demand incubator under alternating temperature of 20 or 30°C and a photoperiod of 8 h (Brasil 2009). The experiment was carried out following a completely randomized design, with 25 seeds per replicate. The following parameters were evaluated: germination (%), GSI, and seedling dry weight (mg seedling^{-1}).

After taking into account variance normality and homogeneity, regression and Kruskal–Wallis test at 5% probability were performed using R software (Team 2020).

3. Results

Chemical analysis revealed that heavy metal values in SS were below the limits established by the CONAMA n° 375 guidelines (Brasil 2006) (Table 1).

Table 1
Chemical and organic composition of SS

Chemical element	Concentration	CONAMA limit (Brasil 2006)
As (mg kg^{-1})	11.2	41
Ba (mg kg^{-1})	518.0	1300
Cd (mg kg^{-1})	1.4	39
Ca (mg kg^{-1})	8221.0	Not specified
Pb (mg kg^{-1})	45.4	300
Cu (mg kg^{-1})	315.0	1500
Cr (mg kg^{-1})	41.1	1000
S (mg kg^{-1})	18306.0	Not specified
Mg (mg kg^{-1})	3761.0	Not specified
Mn (mg kg^{-1})	220.0	Not specified
Hg (mg kg^{-1})	2.3	17
Mo (mg kg^{-1})	1.4	50
N (mg kg^{-1})	5505.0	Not specified
Ni (mg kg^{-1})	20.3	420
K (mg kg^{-1})	6.5	Not specified
Se (mg kg^{-1})	25.3	100
Na (mg kg^{-1})	2764.0	Not specified
Zn (mg kg^{-1})	738.0	2800
P (%)	0.1	Not specified
Organic C (%)	6.5	Not specified
OM (%)	11.3	Not specified
LOM (g kg^{-1} of C)	203.4	Not specified
FFA (g kg^{-1} of C)	0.3	Not specified

Chemical element	Concentration	CONAMA limit (Brasil 2006)
FA (g kg^{-1} of C)	0.1	Not specified
HA (g kg^{-1} of C)	9.8	Not specified
HUMIN (g kg^{-1} of C)	311.13	Not specified
C_{TOTAL} (g kg^{-1} of C)	495.6	Not specified

OM = organic matter; LOM = light-fraction organic matter; FFA = free fulvic acids; FA = fulvic acids; HA = humic acid; HUMIN = humin, residual fraction; C_{TOTAL} = total organic carbon.

Light-fraction organic matter, humic fraction, and total organic carbon were abundant in SS, whereas free fulvic acids and fulvic acid fractions were scarce (Table 1), suggesting that besides providing the soil with sufficient chemicals and nutrients, the sludge introduced large amounts of recalcitrant substances.

The pH of the sludge solutions increased from 7.3 in the absence of SS to 12.4 at a SS concentration of 80 t ha^{-1}, but stabilized thereafter (Fig. 1). Electrical conductivity followed a linear pattern, whereby the maximum value (3521.6 µS cm^{-1} g^{-1}) was observed at a concentration of 520 t ha^{-1} (Fig. 1).

Germination and GSI of *L. sativa* generally decreased with increasing SS concentrations and reached their lowest values at 160, 320, and 520 t ha^{-1} (Fig. 2). A quadratic behavior was observed for root growth, with average values declining beyond 160 t ha^{-1} (Fig. 2B).

L. sativa root growth was four times higher at SS concentration of 160 t ha^{-1} compared to the control, which may be associated with the abundant organic matter and humic fraction in SS (Table 1).

A comparison between *L. sativa* (Fig. 2) and *P. alata*, (Fig. 3) reveals that SS negatively affected seedling germination and initial growth in both species, although the reduction was more pronounced in *P. alata*.

Increasing the SS concentration led to progressive reduction in the mitotic index up to 120 t ha^{-1}; above this concentration, no further changes between quartiles and medians were observed, except at 520 t ha^{-1} (Fig. 4A). The genotoxic effect of SS in *L. sativa* was manifested by an increased frequency of chromosomal aberrations with higher SS concentrations (Fig. 4B). In particular, the aberrations index was 16 times higher at SS concentration of 320 t ha^{-1} compared to that in the negative control, which coincided with a significantly larger number of micronuclei (Fig. 4C).

Genotoxicity at increasing SS concentrations was characterized mainly by a more frequent induction of c-metaphase and occurrence of condensed nuclei (Fig. 5).

4. Discussion

Owing to the complexity of the mixture, it is difficult to determine precisely which compounds are responsible for SS phytotoxicity (Mazzeo et al. 2015). So far, phytotoxicity has been linked primarily to heavy metals (Amin 2011), because they are not easily removed and can be mobilized depending on the physicochemical conditions of the SS and soil in which this residue is applied (Udom et al. 2004). In this study, the concentration of metals in the solid matrix was in accordance with the maximum values established by the Brazilian legislation and previous results (Christofoletti et al. 2013; Martins et al. 2016). These studies show that chemical analyses may not be sufficient to characterize the possible toxic effects of SS because as suggested by Martins et al. (2016), they do not show the synergistic impact of all SS contaminants. The addition of SS increases the extractable form of diethylenetriamine pentaacetic acid, which generates potentially mobile extracts and phytotoxic fractions of heavy metals (Dhanker et al. 2021). Biological tests should complement chemical analyses to better understand the toxic potential of SS (Mazzeo et al. 2015). Moreover, annual and local testing should account for variations in the concentration of heavy metals in each period and location (Gen et al. 2021).

Techniques for heavy metal removal from SS have been tried to ensure the viable and ecologically appropriate use of this substrate in soils. For example, the application of fermentation and bioleaching was tested in anaerobic bioreactors under unregulated pH conditions (acidic or alkaline). In these settings, a combination of ultrasound pretreatment and alkaline fermentation attained the best result, reducing the bioavailability of toxic metals by up to 25% (Yesil et al. 2021). Other tested methods include the application of permeable mesh bags filled with SS and stacked next to corn, banana, and papaya crops. After nine years, more than 94% of heavy metals were found to be retained, the soil had low levels of heavy metals, and those in the harvested fruits were within the designated limits (Lin et al. 2021).

SS can also contain organic compounds with cyto-genotoxic properties, such as organochlorine pesticides, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) (Rank and Nielsen 1998). The adverse biological effects of small organic compounds can be attenuated by their rapid volatilization and biodegradation (Smith 2009). However, Mazzeo et al. (2015) monitored the natural attenuation of a domestic SS and reported high concentrations of the semi-volatile organic compound m- and p-cresol in aqueous extracts and in the crude sludge sample for up to two months. The genotoxic activity of SS samples was detected previously by the chromosome aberrations test in *Allium cepa*. Paraíba et al. (2010) concluded that low-molecular weight PAHs were more likely to accumulate in corn grains than high-molecular weight PAHs. Therefore, SS containing significant concentrations of light PAHs would not be recommended as fertilizers for food crops.

The organic matter of SS in this study was rich in recalcitrant compounds. Although they are highly toxic, some researchers have minimized their contribution to the phytotoxicity of the sludge due to the strong adsorption of these compounds in the solid matrix and, consequently, their lower bioavailability (Smith and Riddell-Black 2007; Smith 2009). However, the effects of these xenobiotics should not be totally neglected, since some of these compounds can be transferred via sludge/soil to plants and eventually, consumers. McLachlan et al. (1994) reported high levels of PCBs and polychlorinated dibenzofuran in

milk from cows grazing in soils fertilized with SS. Therefore, we suggest that organic pollutants, when present at high levels in SS, can contribute to phytotoxicity.

According to Oleszczuk (2012), physicochemical properties determining plant development and growth (such as pH and amount of nutrients) should be taken into account during phytotoxicity analysis of SS. These properties may interfere with plant germination and development and erroneously point to other pollutants. Walter et al. (2006) argued that a combination of several factors contributed to the toxicity of SS, such as concentration and availability of heavy metals, high salinity, and excess of ammonium and organic compounds. In addition, the different toxicity responses of the plants can be attributed to the tolerance of each species or the nature of the sludge. For example, the application of SS (69, 138, and 276 kg of N ha^{-1}) did not influence sunflower (*Helianthus annuus* L.) germination and had a positive influence on morphological characteristics, growth, and seed yield (Koutroubas et al., 2020). In giant miscanthus (*Miscanthus* × *giganteus* Greef and Deuter), SS (160 kg of N ha^{-1}) had a similar effect on yield as mineral fertilizers (Dubis et al. 2020). In industrial hemp (*Cannabis sativa* L.), only 25 t ha^{-1} of SS had a positive effect on plant performance, and beyond that, it had a toxic effect (Praspaliauskas et al. 2020).

The pH range indicated for phytotoxicity studies is between 5.0 and 7.0 (Baumgarten and Spiegel 2004). Dhyèvre et al. (2014) documented that primary roots of *Vicia faba* were necrotized and secondary roots did not exhibit any growth in soil with pH 10.1. Liming helps stabilize SS while also reducing the content of pathogens and augmenting pH (Costa and Costa 2011). However, the stabilized alkaline SS can adversely affect plant growth due to nutritional deficiency (Samaras et al. 2008) from a smaller humic fraction (Ramos et al. 2015). The highest concentrations of sludge exhibited an elevated pH, which can explain the significant inhibition of germination and initial development of *L. sativa* and *P. alata.* This effect was observed with respect to increased electrical conductivity. The soluble salts present in SS can negatively affect the growth of plants, reducing the osmotic potential and compromising the development of the species (Baumgarten and Spiegel 2004).

The results obtained in the present study corroborate those observed by Martins et al. (2016) in *A. cepa* grown in commercial substrate mixed with SS, whereby 28% and 56% reduction in germination was observed at SS concentration of 320 t ha^{-1} and 520 t ha^{-1}, respectively. Oleszczuk et al. (2012) observed that *Sinapis alba* (white mustard) seeds presented germination inhibition of 60% in sandy soil treated with SS (90 t ha^{-1}), whereas Kou et al. (2020) reported that the percentage and germination index of *Brassica campestris* L. ssp. *Chinensis Makino* (Chinese white cabbage) seeds decreased with an increased concentration of sludge, together with increased inhibition of root elongation. In the present study, the deleterious effect of SS addition was more pronounced in *P. alata*. Our data are similar to those observed by Prado and Natale (2005), who concluded that SS, at doses above 10 t ha^{-1}, promoted death of passion fruit due to the presence of metals and excess sodium and potassium ions. In this study, sodium was also present at a very high concentration. Dias et al. (2012) reported that salinity strongly affected passion fruit growth and should be carefully monitored.

SS may also cause disturbances in the plant's cell cycle (Martins et al. 2016). Alterations in cell division can indicate the level of cytotoxicity of environmental pollutants, which compromise metabolic processes, growth, and development (Leme and Marin-Morales 2009; Singh et al. 2009). Faschineto et al. (2007) observed that reduction in the mitotic index coincided with decreased root growth. An increased frequency of interphase cells at SS concentration of up to 120 t ha^{-1} suggests that repair mechanisms lock the cells at this stage to prevent mitosis in cells with possible genetic abnormalities (Palmieri et al. 2014). When repair processes fail, cell death pathways can be activated. Here, the genotoxic effect of elevated SS concentrations was manifested by the presence of condensed nuclei in *L. sativa* root cells (Fig. 5), which led to cell death.

Other commonly observed cell cycle alterations were induction of c-metaphase (84%), followed by the presence of micronuclei (14%), bridges (11%), sticky chromosomes (6%), and chromosome loss (3%). Tests detecting mitotic and chromosomal abnormalities help identify the mechanism of action employed by toxic compounds. These may be clastogenic, with abnormalities, such as chromosome breakage and bridges or aneugenic, leading to anomalies, such as chromosomal losses, c-metaphase, and sticky chromosomes (Leme and Marin-Morales 2009; Rossato et al. 2010). It is possible to infer that the studied SS concentrations employed both aneugenic and clastogenic mechanisms of action (Fig. 5), although the former were prevalent. These results are similar to those found by Amin et al. (2009), who studied *V. faba* meristem cells and found changes in the mitotic spindle after consecutive SS applications to the soil. Moreover, Amin (2011) demonstrated the occurrence of aneugenic effects in meiocytes of *Zea mays* after exposure to SS.

A micronucleus can be formed by chromosomal fragments or by the loss of entire chromosomes. As no breakage was observed in this study, it appears that micronuclei were formed by the loss of entire chromosomes, further confirming the aneugenic effect of SS.

The increase in cyto-genotoxicity observed in root meristem cells of *L. sativa* as a function of increased pH and addition of SS corroborates the results of Dhyèvre et al. (2014). According to the authors, under extreme conditions of pH (3.5, 4.3, and 9.0) root cells of *V. faba* exposed to soil contaminated with 5 µg kg^{-1} maleic hydrazine showed a higher frequency of micronuclei and a lower mitotic index. At the highest tested concentrations of maleic hydrazine, the cell proliferation rate decreased dramatically, indicating double stress, i.e., extreme pH and xenobiotic concentration.

5. Conclusion

The results obtained in the present study suggest that pH and electrical conductivity increase simultaneously with the application of higher SS doses. SS concentrations above 120 t ha^{-1} adversely affect germination and early seedling growth of *L. sativa* and *P. alata*. At higher concentrations (120 t ha^{-1}), SS induces genetic lesions in *L. sativa*, plus chromosomal and nuclear alterations. *L. sativa* root cells treated with SS concentration of 520 t ha^{-1} were broken, which was attributed to the toxicity caused by an excess amount of sludge and prevented their mitotic and chromosome alterations analysis. The

present study highlights the importance of investigating the optimal SS dosage for crops to ensure maximal growth and minimal toxicity.

Abbreviations

GSI, germination speed index; PAH, polycyclic aromatic hydrocarbon; SS, sewage sludge; PCBs: polychlorinated biphenyls

Declarations

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Consent to Publish

All of authors provided their consent for the publication of this manuscript.

Author Contributions Statement

ARF conceptualization, preparation of experiments, investigation, writing, editing. NABF preparation of experiments, investigation. RSA writing - review and editing, supervision. TSS preparation of experiments, critical review of important intellectual content. ING preparation of experiments, critical review of important intellectual content. JOB critical review of important intellectual content. PAML data curation, software, re-analysis, preview. TM writing, and review editing. WCO writing, review. JCL writing - review and editing, supervision, resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

1. ABNT - Associação Brasileira de Normas Técnicas (2004) Amostragem de resíduos sólidos. NBR 10007. ABNT, Rio de Janeiro.
2. Amin AW, Sherif FK, El-Atar E, Ez-Eldin H (2009) Effect of residual and accumulative sewage sludge on heavy metals bioaccumulation: gene action and some yield parameters of *Vicia faba*. Res J Environ Toxicol 3:60–75.
3. Amin AW (2011) Evaluation of the genotoxicity of residual repeated applications of sewage sludge on M2 meiocytes of *Zea* plants. Res J Environ Toxicol 5:235–250.
4. Andrade LF, Davide LC, Gedraite LS (2010) The effect of cyanide compounds, fluorides, aluminum, and inorganic oxides present in spent pot liner on germination and root tip cells of *Lactuca sativa*. Ecotox Environ Safe 73:626–631.
5. Baumgarten A, Spiegel H (2004) Phytotoxicity (Plant tolerance). Agency for Health and Food Safety, Vienna, 1–36.
6. Brasil, Conselho Nacional do Meio Ambiente, Conama (2006) Resolução, N° 375: Define critérios e procedimentos, para o uso agrícola de lodos de esgoto gerados em estações de tratamento de esgoto sanitário e seus produtos derivados, e dá outras providências, one hundred and sixty seven eds. Diário Oficial Da União, Brasília, Brasil.
7. Brasil-Ministério da Agricultura, Pecuária e Abastecimento (2009) Regras para análise de sementes. MAPA/ACS, Brasília.
8. Chow HY, Pan M (2020) Fertilization value of biosolids on nutrient accumulation and environmental risks to agricultural plants. Water Air Soil Pollut 231 (578):1–13.
9. Christofoletti CA, Pedro-Escher J, Fontanetti CS (2013) Assessment of the genotoxicity of two agricultural residues after processing by diplopods using the *Allium cepa* assay. Water Air Soil Pollut 224:1–14.
10. Chu L, He W (2021) Toxic metals in soil due to the land application of sewage sludge in China: Spatiotemporal variations and influencing factors. Sci Total Environ 757:143813.
11. Costa AN, Costa AFS (2011) Manual de uso agrícola e disposição do lodo de esgoto para o estado do Espírito Santo, first ed, Vitória: Incaper.
12. Dhanker R, Chaudhary S, Goyal S, Garg VK (2021) Influence of urban sewage sludge amendment on agricultural soil parameters. Environ Technol Innov 23:101642.
13. Dhyèvre A, Foltête AS, Muller DAS, Cotelle S (2014) Effects of soil pH on the *Vicia*-micronucleus genotoxicity assay. Mutat Res 774:17–21.
14. Dias JT, Sa RJ, Silva MNB, Dantas SAG, Cavalcante LF, Campos VB, Miguel AA (2012) Crescimento das plantas de maracujazeiro amarelo após o transplantio, in: Cavalcante, L.F., (Ed.), O maracujazeiro amarelo e a salinidade da água. João Pessoa, Sal da Terra, pp. 97–137.
15. Dubis B, Jankowski KJ, Zaluski D, Sokólski M (2020) The effect of sewage sludge fertilization on the biomass yield of giant miscanthus and the energy balance of the production process. Energy

154:118189.

16. Faschineto JM, Bagatini MD, Durigon J, Silva ACF, Tedesco SB (2007) Efeito antiproliferativo das infusões de *Achyrocline satureioides* DC (Asteraceae) sobre o ciclo celular de *Allium cepa*. Rev Bras Farmacol 17:49–54.
17. Felicidade I, Lima JD, Pesarini JR, Monreal ACD, Mantovani MS, Ribeiro LR, Oliveira RJ (2014) Mutagenic and antimutagenic effects of aqueous extract of rosemary (*Rosmarinus officinalis* L.) on meristematic cells of *Allium cepa*. Gen Mol Res 13:9986–9996.
18. Freitas AR, Lopes, JC, Alexandre RS, Venancio LP, Zanotti RF (2015) Emergência e crescimento de mudas de maracujá doce em função de lodo de esgoto e luz. Comun Sci 2:234–240.
19. Grant WF (1999) Higher plant assays for the detection of chromosomal aberrations and gene mutations-a brief historical background on their use for screening and monitoring environmental chemicals. Mutat Res 426:107–112.
20. Grotto D, Carneiro MFH, Sauer E, Garcia SC, Melo WJ, Barbosa JRF (2013) Evaluation of biochemical and redox parameters in rats fed with corn grown in soil amended with urban sewage sludge. Ecotox Environ Safe 95:188–194.
21. Hoshina MM, Marin-Morales MA (2009) Micronucleus and chromosome aberrations induced in onion (*Allium cepa*) by a petroleum refinery effluent and by river water that receives this effluent. Ecotox Environ Safe 72:2090–2095.
22. IHSS - International Humic Substances Society (2016) Products. Available in: <http://www.ihss.gatech.edu/products.html >. Accessed in: 10 October 2016.
23. Kelessidis A, Stasinakis AS (2012) Comparative study of the methods used for treatment and final disposal of sewage sludge in European countries. Wast Manag 32:1186–1195.
24. Kou Y, Zhao Q, Cheng Y, Wu Y, Dou W (2020) Removal of heavy metals in sludge via joint EDTA-acid treatment: Effects on seed germination. Sci Total Environ 707:135866.
25. Koutroubas SD, Antoniadis V, Damalas CA, Fotiadis S (2020) Sunflower growth and yield response to sewage sludge application under contrasting water availability conditions. Ind Crops Prod 154:112670.
26. Leme DM, Marin-Morales MA (2009) *Allium cepa* test in environmental monitoring: A review on its application. Mutat Res 82:71–81.
27. Lin X, Li S, Wei Z, Chen Y, Hei L, Wu Q (2021) Indirect application of sludge for recycling in agriculture to minimize heavy metal contamination of soil. Resour Conserv Recy 166:105358.
28. Maguire JD (1962) Speed of germination-aid in selection and evaluation for seedling emergence and vigor. Crop Science 2:176–177.
29. Markowicz A, Bondarczuk K, Wiekiera A, Sutowicz S (2021) Is sewage sludge a valuable fertilizer? A soil microbiome and resistome study under field conditions. J Soils Sediments 21:2882–2895.
30. Martins MNC, Souza VV, Souza TS (2016) Cytotoxic, genotoxic and mutagenic effects of sewage sludge on *Allium cepa*. Chemosphere 148:481–486.

31. Mazzeo DEC, Fernandes TCC, Levy CE, Fontanetti CS, Marin-Morales MA (2015) Monitoring the natural attenuation of a sewage sludge toxicity using the *Allium cepa* test. Ecol Indic 56:60–69.
32. McLachlan MS, Hinkel M, Reissinger M, Hippelein M, Kaupp H (1994) A study of the influence of sewage sludge fertilization on the concentrations of PCDD/F and PCB in soil and milk. Environ Pollut 85:337–343.
33. Nagajyoti PC, Lee KD, Sreekanth TVM (2010) Heavy metals, occurrence and toxicity for plants: a review. Environ Chemy Lett 8:199–216.
34. Nascimento AL, Sampaio RA, Zuba Junio GR, Fernandes LA, Cruz SF, Carneiro JP, Barbosa CF, Lima NN (2014) Atributos químicos do solo adubado com lodo de esgoto estabilizado por diferentes processos e cultivado com girassol. Biosc J 30:146–153.
35. Oleszczuk P, Malara A, Josko I, Lesiuk A (2012) The phytotoxicity changes of sewage sludge-amended soils. Water Air Soil Pollut 223:4937–4948.
36. Paraíba LC, Queiroz SCN, Maia AHN, Ferracini VL (2010) Bioconcentration factor estimates of polycyclic aromatic hydrocarbons in grains of corn plants cultivated in soils treated with sewage sludge. Sci Tot Environ 408:3270–3276.
37. Palmieri MJ, Luber J, Andrade-Vieira LF, Davide LC (2014) Cytotoxic and phytotoxic effects of the main chemical components of spent pot-liner: A comparative approach. Mutat Res 763:30–35.
38. Prado RM, Natale W (2005) Desenvolvimento inicial e estado nutricional do maracujazeiro em resposta à aplicação de lodo têxtil. Pesq Agr Bras 40:621–626.
39. Praspaliauskas M, Žaltauskaitė J, Pedišius N, Striūgas N (2020) Comprehensive evaluation of sewage sludge and sewage sludge char soil amendment impact on the industrial hemp growth performance and heavy metal accumulation. Ind Crops Prod 150:112396.
40. Ramos AC, Olivares FL, Silva LS, Aguiar NO, Canellas LP (2015) Humic matter elicits proton and calcium fluxes and signaling dependent on Ca^{2+} dependent protein kinase (CDPK) at early stages of lateral plant root development. Chem Biol Tech Agr 2:1–12.
41. Rank J, Nielsen MH (1998) Genotoxicity testing of wastewater sludge using the *Allium cepa* anaphase-telophase chromosome aberration assay. Mutat Res 418:113–119.
42. Rossato LV, Tedesco SB, Laughinghouse HD, Farias JG, Nicoloso FR (2010) Alterations in the mitotic index of *Allium cepa* induced by infusions of *Pluchea sagittalis* submitted to three different cultivation systems. An Acad Bras Ciênc 82:857–860.
43. Santos JS, Pontes MS, Nobrega MAS, Santiago EF (2021) Effects of substrates containing different concentrations of sewage sludge on physiological parameters and quality of *Alibertia edulis* (Rubiaceae) seedlings. Environ Sci Pollut Res 28:25984–25992.
44. Taiz L, Zeiger E, Møller IM, Murphy A (2017) Fisiologia e desenvolvimento vegetal. 6ª ed. - Porto Alegre: Artmed, 858 p.
45. TEAM RC (2020) A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2017. Disponível em: <https://www.rproject.org/>. Acesso em: 10 fev. 2020.

46. Thurman EM, Malcolm RL (1981) Preparative isolation of aquatic humic substances. Environ Sci Technol 15(4):463–466.
47. Samaras P, Papadimitriou CA, Papastergiadis E, Pappa A, Gudulas K (2008) Study of phytotoxic properties of sewage sludge stabilised by alkaline mediums, WIT Trans. Ecol Environ 110:93–100.
48. Singh BN, Singh BR, Singh RL, Prakash D, Singh DP, Sarma BK, Upadhyay G, Singh HB (2009) Polyphenolics from various extracts/fractions of red onion (*Allium cepa*) peel with potent antioxidant and antimutagenic activities. Food Chem Toxicol 47:1161–1167.
49. Singh RP, Agrawal M (2008) Potential benefits and risks of land application of sewage sludge. Wast Manag 28:347–358.
50. Smith SR, Riddell-Black D (2007) Sources and impacts of past, current and future contaminantion of soil. Appendix 2: organic contaminants, 2007, 1-235 (http://www.imperial.ac.uk/ewre) (accessed 10.07.17).
51. Smith, S.R., 2009. Organic contaminants in sewage sludge (biosolids) and their significance for agricultural recycling. Philos. Trans. R. Soc. A367, 4005–4041.
52. Sousa SM, Silva OS, Viccini LF (2010) Cytogenotoxicity of *Cymbopogon citratus* (DC) stapf (lemon grass) aqueous extracts in vegetal test systems. An Acad Bras Ciênc (82)305–311.
53. Taiz L, Zeiger E, Moller IM, Murphy A (2015) Plant Physiology and Development. Sinauer Associates, sixth ed., Sunderland, ING.
54. Thurman EM, Malcolm RL (1981) Preparative isolation of aquatic humic substances. Environ Sci Tech 15:463–466.
55. Udom BE, Mbagwu JSC, Adesodun JK, Agbim NN (2004) Distributions of zinc, copper, cadmium and lead in a tropical ultisol after long-term disposal of sewage sludge. Environ Inter 30:467–470.
56. Walter I, Martinez F, Cala V (2006) Heavy metal speciation and phytotoxic effects of three representative biosolids for agricultural uses. Environ Pollut 139:507–514.
57. Yesil H, Molaey R, Calli B, Tugtas AE (2021) Removal and recovery of heavy metals from sewage sludge via three-stage integrated process. Chemosphere 280:130650.

Figures

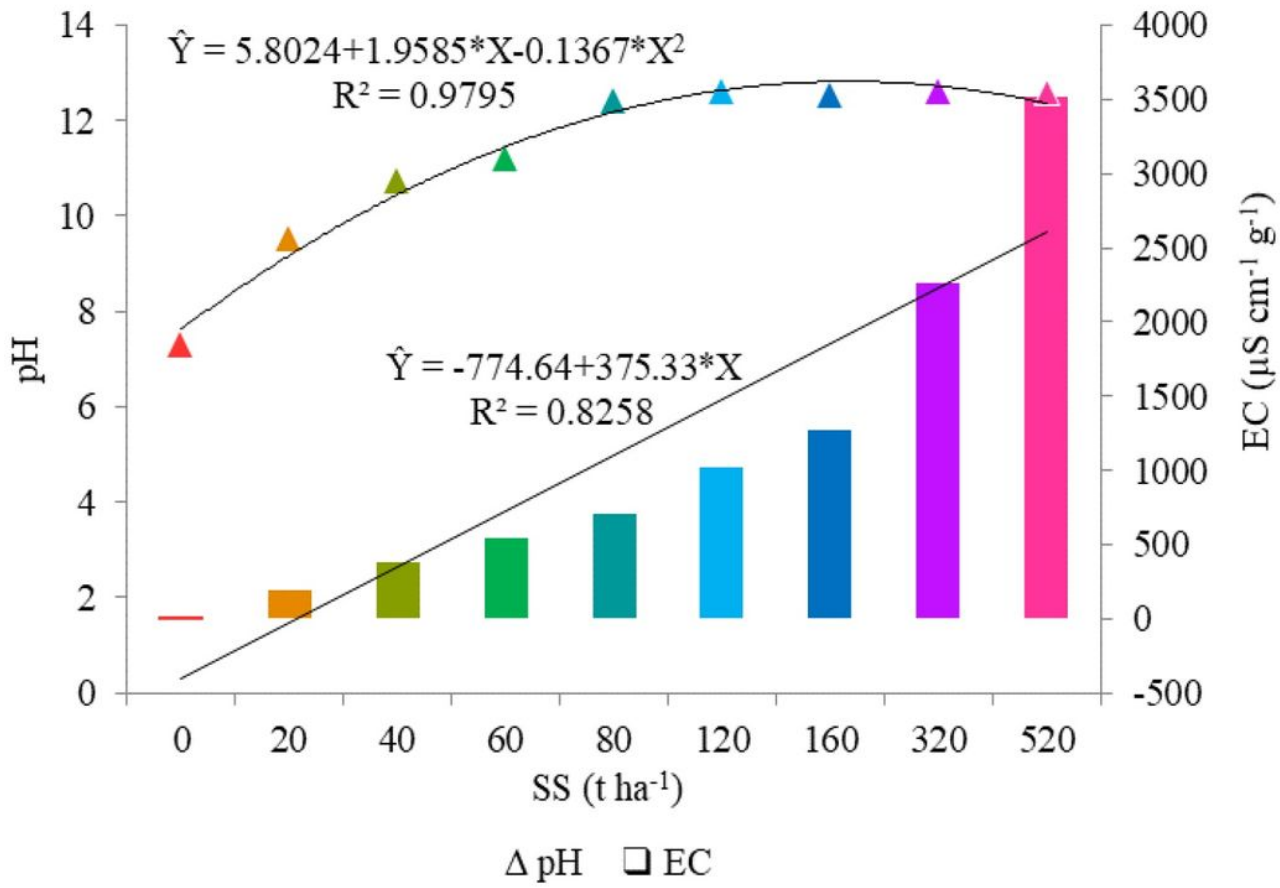

Figure 1

Dependence of pH and electrical conductivity (EC) on SS concentration in sludge solutions. *Significant difference at 5%.

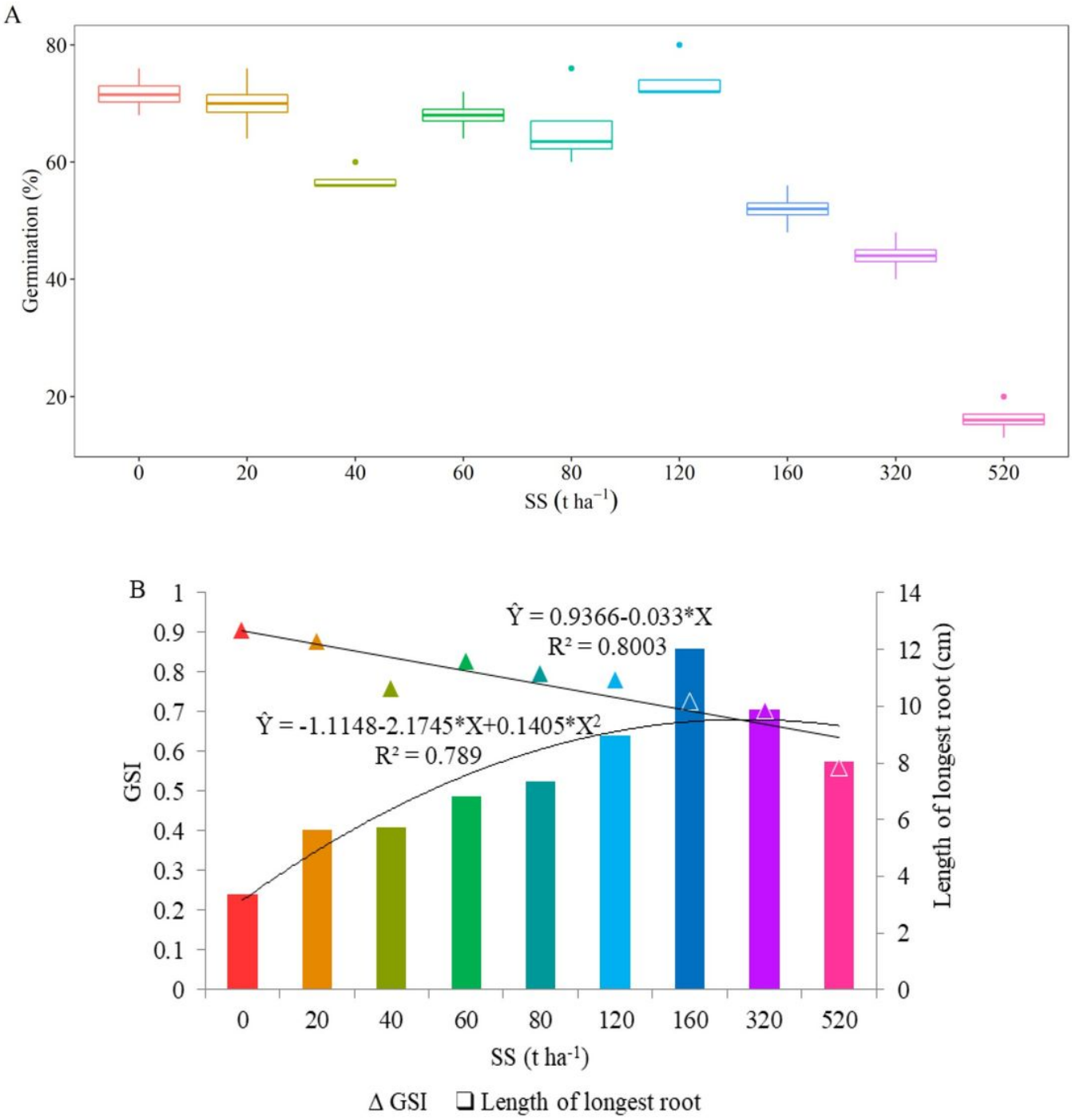

Figure 2

(**A**) Box-plot of germination (%) of *L. sativa*relative to SS concentration as determined by the Kruskal-Wallis test. (**B**) Germination speed index (GSI) and root length of *L. sativa* relative to SS concentration. Regression analyses results are shown. *Significant difference at 5%.

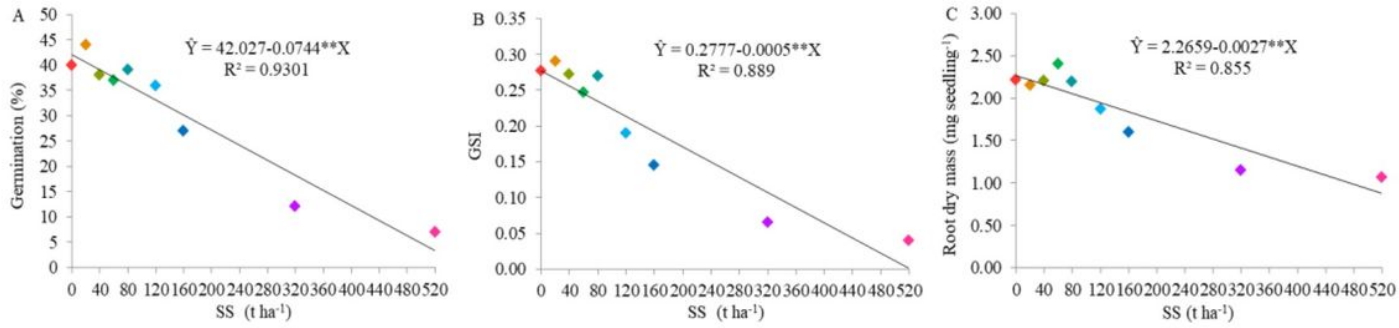

Figure 3

Germination (%), germination speed index (GSI), and root dry mass (mg seedling^{-1}) of *P. alata* at different concentrations of SS. **Significant difference at 1%.

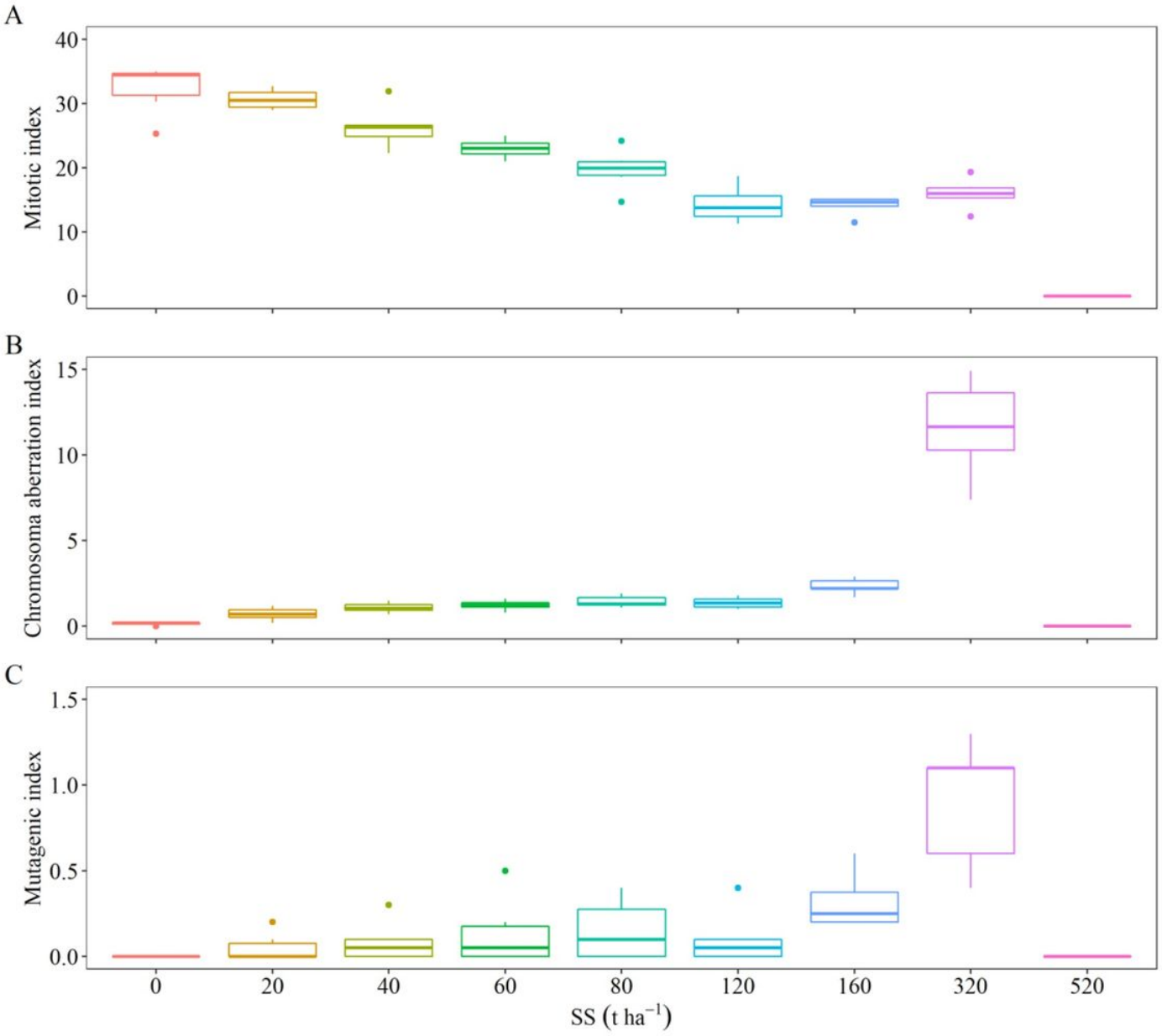

Figure 4

Box-plots of mitotic index (**A**), chromosomal aberration index (**B**), and mutagenic index (**C**) in *L. sativa*cells relative to different SS concentrations. Values were calculated using the Kruskal-Wallis test at 5% probability.

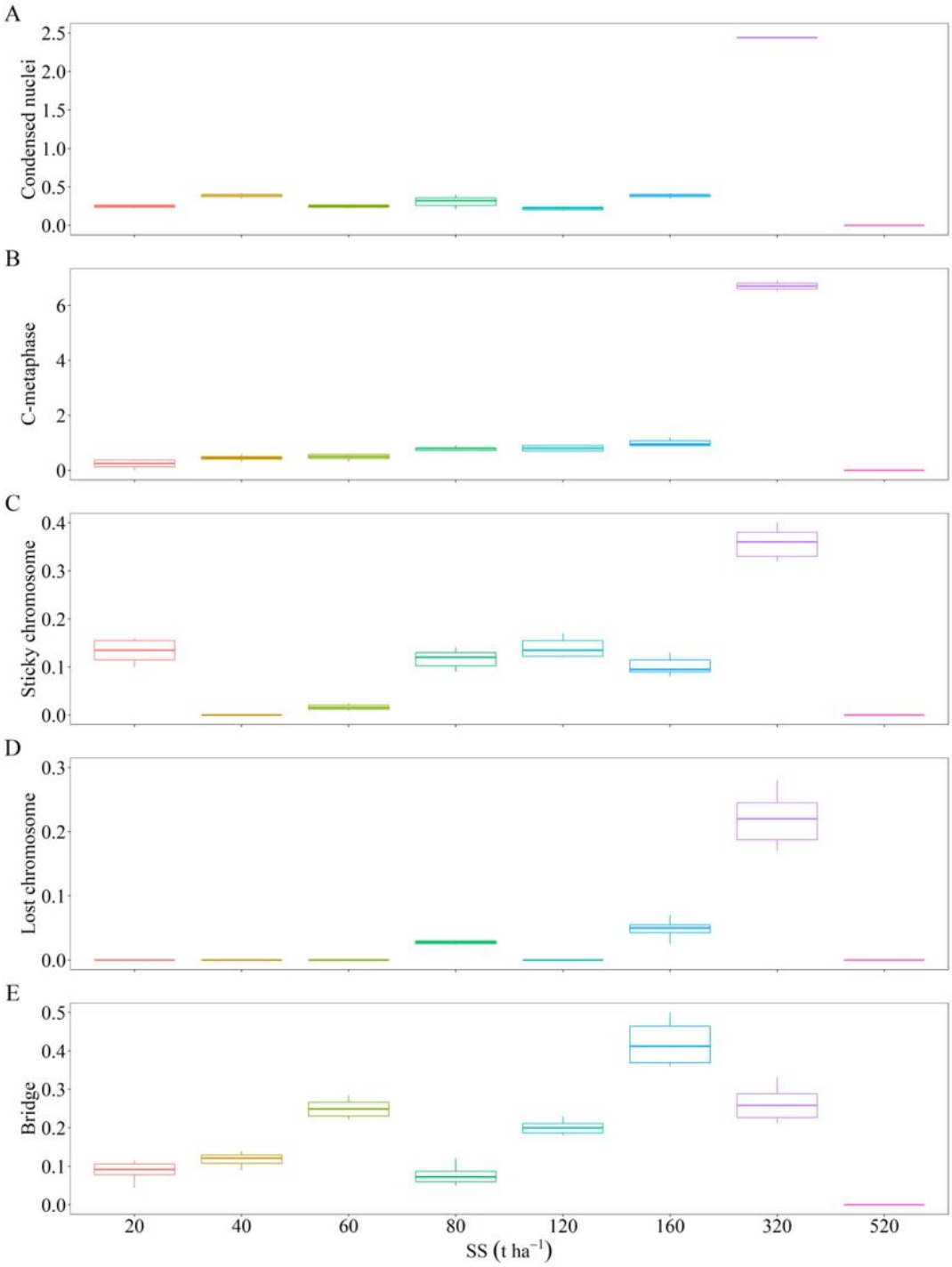

Figure 5

Box-plots of condensed nuclei (**A**), c-metaphase (**B**), sticky chromosomes (**C**), lost chromosomes (**D**), and bridges (**E**) in meristem cells of *L. sativa*relative to different SS concentrations. Values were calculated using the Kruskal-Wallis test at 5% probability.