In Vitro Regeneration of Ragleaf (*Crassocephalum crepidioides* (Benth.) S.Moore) Using Kinetin

10.18196/pt.v12i1.20022

Murtadha, Mosobalaje Abdulsalam*, Oladejo, Omobolanle Olanike

Department of Agronomy, Faculty of Agricultural Production and Management, College of Agriculture, Osun State University, Ejigbo, Nigeria

*Corresponding author: mosobalaje.murtadha@uniosun.edu.ng

ABSTRACT

Micropropagation is a valuable means for large-scale production and preservation of invasive alien species such as ragleaf. To determine the concentration of kinetin adequate for *in vitro* growth of the ragleaf flower, seeds of the plant were propagated *in vitro* using Murashige and Skoog medium supplemented with basal 0.5 mg L^{-1} Naphthalene Acetic Acid and incubated for four weeks. The growing shoots were cut into pieces and inoculated into MS media with five kinetin concentrations: 0.0, 1.0, 1.5, 2.0, and 2.5 mg L^{-1}. The tubes were kept in an incubation room for seven weeks. The experiment was arranged in a completely randomized design with ten replications. Shoot length (cm) and the number of leaves, nodes, and roots were measured. The control medium recorded less growth performance across all the variables, indicating the important role of the exogenous kinetin. The plants in 2.50 mg L^{-1} kinetin were the tallest (7.23±0.87 cm) with the highest number of leaves (28.17±2.57), nodes (10.50±1.02) and shoots (3.83±0.17). The media containing 2.50 mg L^{-1} kinetin also produced the highest number of roots (9.00), which is necessary for the survival of the seedlings. The results indicate that 2.5 mg L^{-1} is adequate for cultured *C. crepidioides*.

Keywords: Kinetin; Micro-propagation; Node; Root; Shoot

INTRODUCTION

Crassocephalum crepidioides (redflower, ragleaf, thickhead, fireweed) is a member of the Asteraceae family, an aggressive, invasive herb in the global compendium of weeds (Randall, 2017). However, in tropical Africa and Asia (Bajgai et al., 2023), the tender, succulent leaves and stems of *C. crepidioides* are consumed, sold, and used for healing purposes or as green fodder for poultry and other livestock (Denton, 2014). It thus contributes immensely to the economy of rural families in the region, where 25–27 t ha^{-1} of leaves and shoots are collected annually. In addition, the herb is notably recognized for its high protein content and medicinal and various health benefits (Nguyen & Dang, 2020; Oyebode et al., 2020). There is, however, ample evidence that *C. crepidioides* can accumulate in the shoot a high concentration of toxic compounds known as pyrrolizidine alkaloid (PA) jacobine when nitrogen is deficient in the soil, and efforts are on to develop PA-free cultivars (Schramm et al., 2021).

Naturally, the plant exists in the rainforest region of Nigeria, and it is consumed as a vegetable in soup. Through the ages, the plant conserves its species through seed dispersal and germination during

Check for updates
open access
Article History
Received: 24 September 2023
Accepted: 15 February 2024

the rainy season without human intervention. Its availability in the market is thus limited to the little quantity farmers can collect from the wild. However, a yield ranging from 25 to 27 t ha^{-1} of leaves and shoots of ragleaf is produced annually from harvest (Denton, 2014). Conventional vegetative propagation of ragleaf is less successful, as the plant rapidly enters the flowering stage shortly after transplanting. Other production constraints include seed dispersal from immature and malformed fruits, limitation of seed dispersal ability (Intanon et al., 2020), photoblastic seed germination (Yuan & Wen, 2018), and loss of seed viability under prolonged storage. Several solutions were suggested, including breeding improved cultivars and producing viable seeds. Despite the possible sustainability of these opinions, little progress, except that of Omonhinmin et al. (2023), has been achieved because most vegetable researchers and breeders consider the crop unimportant; and available genetic variation has not been documented nor exploited to date.

In vitro multiplication has been used to solve some constraints in the propagation of various plants. When applied to plant leaves, a review of the roles of plant growth regulators (PGRs) was provided (Sosnowski et al., 2023). The first stage in mass propagating ragleaf is determining the proper concentration of plant growth hormones, such as kinetin, for shoot and root induction. Kinetin, a synthetic cytokinin PGR, plays a crucial role in tissue culture, mainly, in promoting cell division and induction of meristematic cells, which are the precursors of shoot formation (Xiong et al.; 2022; Vaishnav & Chowdhury, 2023). In their report, Hairuddin et al. (2023) reported positive roles of auxin and cytokinin in maize under *in vitro* micropropagation. Kane (2005) stressed that Naphthalene Acetic Acid (NAA) at 0.01 to 10 mg/L was the most widely used and effective plant growth regulator for root induction. The second stage of mass propagating ragleaf is to determine which plant part responds more to the micropropagation of the plant. Salami (2019) determined a 15 cm seedling height as the optimum planting height (regeneration potential) to regenerate *C. crepidioides*; the treatment was performed excellently across the parameters examined (plant height, stem girth, number of branches, and leaf area). It was also found that arachidonic acid and eicosapentaenoic acid were higher in 25 mg L^{-1} and 15 mg L^{-1} of kinetin compared with control in an *in vitro* study of *Tetraselmis suecica* (Asghari et al., 2023).

There has been interest in *C. crepidioides* as bioremediation plants, medicinal herbs, and others (Schramm et al., 2021; Mudau et al., 2022); however, scanty reports on *in vitro* propagation of *C. crepidioides* are available in the literature. The method has been applied to other important plants. Mohamad et al. (2022) stated that shoot explants increased with benzyl adenine and kinetin for micropropagation of *Paulownia spp*. Bulya et al. (2023) reported the growth of callus in medicinal plants cultured in media containing kinetin and no growth in media without kinetin, implying the importance of this hormone for callus development. Khan et al. (2021) and Kaviani et al. (2023) showed that PGR could induce roots in pear rootstock and medicinal plants (*Saussurea costus*) propagated *in vitro*.

Generally, *in vitro* regeneration remains the most appropriate means to alleviate some of the production and conservation problems of *C. crepidioides*. *In vitro* multiplication with the right hormone concentration coupled with sub-cultured seed explant is expected to be a successful micropropagation protocol for this plant. A single seed, when sub-cultured, has the advantage of giving thousands of

new plants, thereby offering a chance for mass propagation of plants from a single source. Therefore, this study aimed to determine the concentration of kinetin and the possibility of obtaining multi-plants from subculturing a single seed of *C. crepidioides.* The results of this study will proffer solutions to the problem of mass production of ragleaf seedlings by suggesting a protocol for culture, thereby assisting in the maintenance of improved selected lines or cultivars for commercial production.

MATERIALS AND METHODS

Collection and Sterilization of Propagules

The experiment was conducted at the Tissue Culture Laboratory of the National Centre for Genetic Resources and Biotechnology (NACGRAB), Apata, Ibadan, Oyo State. Seeds of mature plants of *C. crepidioides* were collected from the field gene bank, washed in a liquid detergent for 3 min, rinsed in sterile distilled water for 20 min, soaked using 70% ethanol for 5 min, and lastly, soaked in 20% and 10% sodium hypochlorite for 20 and 10 min, respectively. The seeds were then rinsed three times with distilled water, as Gopi & Vatsala (2006) suggested.

Culture medium preparation and explant inoculation

Basal medium (MS) was prepared following the procedure of Murashige & Skoog (1962) by adding macro and micronutrients, sucrose, vitamins, iron, EDTA, inositol, and agar to support the growth of the explant. All materials and equipment were sterilized prior to autoclaving. The pH of the formulated medium was adjusted to 5.7 by adding 1 molar (IM) of HCl to 0.7% agar (Difco, USA). Naphthalene Acetic Acid was added to the MS medium at a concentration of 0.5 mg L^{-1} as base PGR. The medium was prepared a day before culturing and kept inside the growth chamber to solidify. Three sterilized seeds were inoculated into 10 test tubes with 10 ml medium under a laminar flow hood using forceps and blades. The culture was transferred to the growth room for four weeks to enable seed germination. Afterward, the shoots of the germinated seeds were fragmented, and a 1-1.5 cm fragment was hygienically inoculated into the MS medium supplemented with kinetin at concentrations of 1.0, 1.5, 2.0, and 2.5 mg L^{-1} in glass tubes, with control being without kinetin in ten replicates. The glass tubes were sealed with paraffin wax, labeled accordingly, and kept in an incubator at 26±1°C with a photoperiod of 24 h in the growth room for eleven weeks. Contaminated growth tubes were isolated and removed from the incubation room.

Experimental design and data analysis

The design of the experiment followed a Completely Randomized Design (CRD), with kinetin treatment being the factor. Data were recorded weekly commencing from the first week after incubation till the eleventh week on variables of shoot length (cm), number of leaves, number of nodes, and shoot number, but the number of roots per seedling was recorded at the eleventh week only. The recorded data were subjected to variance analysis using statistical analysis software SAS version 9.4. The variables that showed differences at the probability level of $p \leq 0.05$ were considered significant and compared by the Least Significant Difference (LSD) at 5% probability. Microsoft Excel was used to present growth development graphically.

RESULTS AND DISCUSSION

Kinetin significantly affects the germination of *C. crepidioides* seed sub-cultured *in vitro* (Table 1). Media with kinetin concentration 2.5 significantly recorded the highest values for shoot length (cm), number of leaves, number of nodes, number of shoots, and number of roots, while the least was recorded in media without the plant growth hormone. The growth performance of *C. crepidioides* observed in MS media supplemented with kinetin suggested the supplied cytokinin's active role and participation in the regeneration of shoots, leaves, nodes, and roots. This improvement in growth parameters at high kinetin concentrations is attributable to the positive role of cytokinin and its possible interaction with NAA, which is known to stimulate cell division and reduce lateral bud dormancy. Hairuddin et al. (2023) reported a similar observation in maize. The increase in shoot length is also due to the effects of the phytohormones (auxin and cytokinin) on the initiation of cell division, cell growth, and expansion. The lower values of the measured organs in the control treatment revealed that the tissue might contain natural phytohormones but not enough to ensure the huge *in vitro* growth of ragleaf organs.

Table 1. Effects of different kinetin concentrations on the *in vitro* growth of *C. crepidioides*

Kinetin Concentration mg L^{-1}	Plantlet Height (cm)	Leaf Number	Number of nodes	Number of shoots	Number of roots
0.00	0.93±0.04^{c}	1.67±0.33^{c}	1.83±1.08^{b}	1.00±0.00^{c}	2.50±0.05^{b}
1.00	2.48±0.54^{bc}	5.25±1.84^{c}	3.50±1.32^{b}	1.75±0.25^{bc}	2.00±0.00^{b}
1.50	1.83±0.27^{c}	6.25±0.25^{c}	3.00±1.08^{b}	2.00±0.41^{b}	4.00±0.00^{ab}
2.00	3.96±0.29^{b}	15.95±1.27^{b}	5.37±0.77^{b}	2.11±0.21^{b}	5.83±0.98^{ab}
2.50	7.23±0.87^{a}	28.17±2.57^{a}	10.50±1.02^{a}	3.83±0.17^{a}	9.00±1.00^{a}

Remarks: ± standard error of the mean
Means along the same column with the same alphabets are not significantly different at $P < 0.05$.

In Figures 1a, b, c, and d, shoot length (cm), number of leaves, number of nodes, and number of shoots had more rapid development in culture media containing kinetin than in media without kinetin. This type of growth performance of *C. crepidioides,* as observed in MS medium supplemented with PGR, suggested the active role of supplied cytokinin and its participation in the regeneration of the organs. The improvement in concentrations is attributable to the role of cytokinin and auxin (alone or together), which is known to stimulate cell division (Asghari et al., 2023) and reduce lateral bud dormancy in plant tissue culture (Davies, 2004). The increase in shoot length is also due to the effects of the phytohormone influencing the initiation of cell division with cell growth and expansion. The performance displayed by C. c*repidioides* MS media enriched with kinetin 2.5 mg L^{-1} proves that a high concentration of kinetin is necessary for the survival of *C. crepioides*. Similar reports were given by Bulya et al. (2023) and Hairuddin et al. (2023) in maize and medicinal plants, respectively. The weekly increase in these parameters further suggests that kinetin at higher concentrations tends to increase the growth of plantlets positively, and this corroborated the report of Vaishnav & Chowdhury (2023), who observed that in a hormone-free medium, the rate of growth was very poor. Although PGR media has been helpful in the growth of this plant, there are reports of other members of Asteraceae that developed in a medium without PGRs. In these cases, endogenous auxin was implicated in shoot regeneration and multiplication.

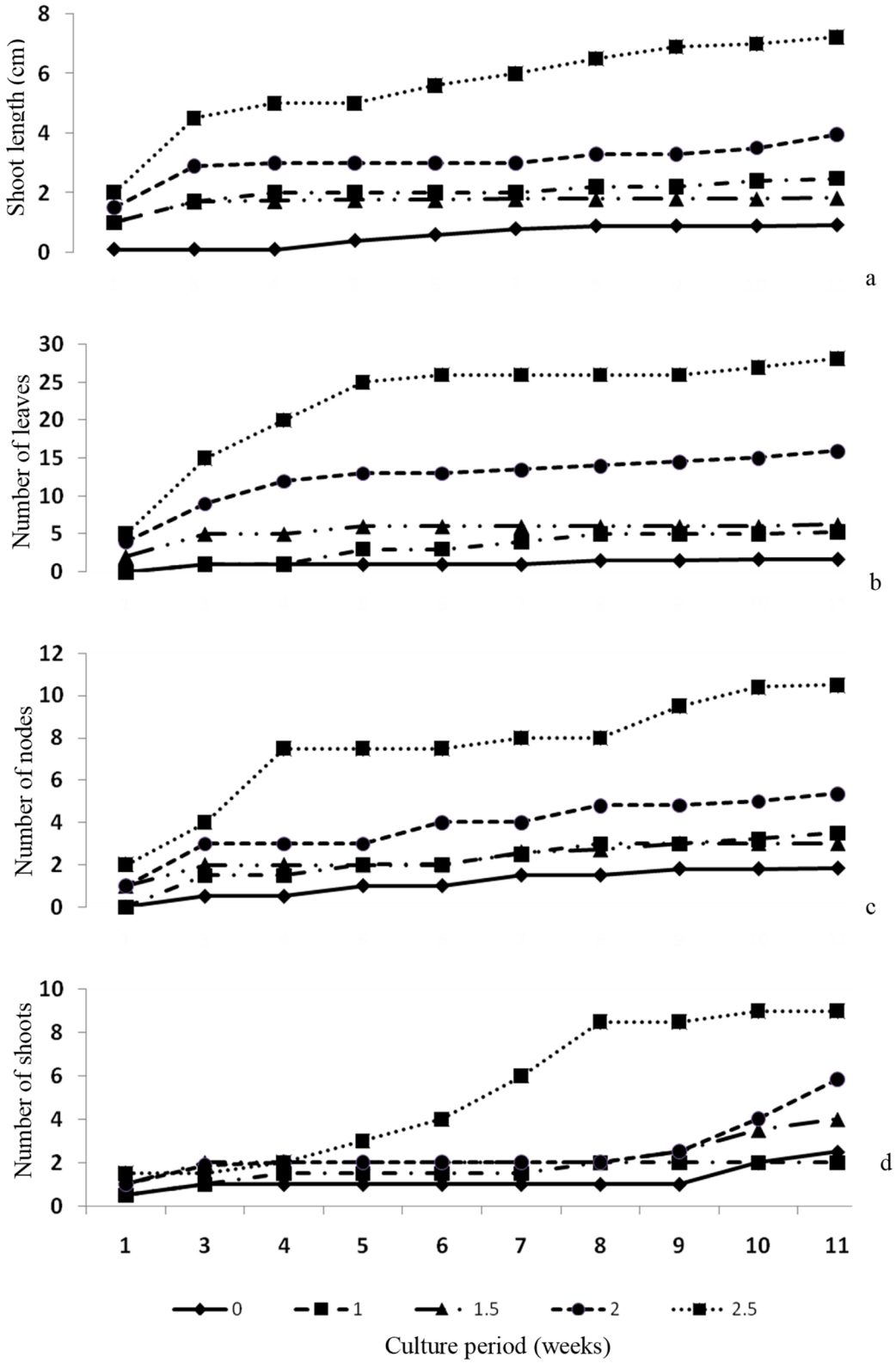

Figure 1. In vitro growth of *C. crepidioides* under difference kinetin concentrations (0, 1.0, 1.5, 2.0, 2.5 mgL^{-1}) for 11 weeks of treatment, a) shoot length (cm) b) number of leaves, c) number of nodes, and d) number of shoots

The insignificance of the number of roots observed among the cultures enriched with kinetin suggests that the hormone is vital for seedling survival in the late stages of propagation. This is obvious in Figure 2, which displays various samples of plantlets and massive growth of shoots and roots of *C. crepidiodes* when MS was supplemented with 2.5 mg L^{-1} kinetin. This result is supported by earlier studies by Kaviani et al. (2023) that showed that shoot proliferation was best achieved with a combination of Butyric acid (BA) and Kinetin, while MS medium supplemented with Indole-3-acetic acid (IAA) enhanced roots proliferation. Also, Khan et al. (2021) found the best root growth in a similar condition of MS medium.

a. b.

Figure 2. *C. crepidioides* in MS media enriched with 2.5 mg L^{-1} kinetin; a) different samples of plantlets. b) flagleaf shoots and roots development

CONCLUSION

Supplementing MS medium with kinetin is important for high growth performance and mass propagation of *C. crepidioides*. MS media with kinetin (2.50 mg L^{-1}) is needed to achieve better growth of cultured ragleaf.

ACKNOWLEDGMENTS

The authors wish to thank the management and staff of the National Center for Genetic Resources and Biotechnology (NACGRAB) for their invaluable contributions to the success of this project. We also appreciate the management of the College of Agriculture, Osun State University, for providing some of the materials used in this project.

REFERENCES

Asghari, T., Ahmadifard, N., & Nikoo, M. (2023). Effect of Phytohormone Kinetin on Cell Density, Photosynthetic Pigments, Antioxidant Enzymes, and Fatty Acid Composition of the Microalgae *Tetraselmis suecica*. *Aquaculture Research*, *2023*. https://doi.org/10.1155/2023/5967771

Bajgai, R. C., Bajgai, Y., & Johnson, S. B. (2023). The presence of wild edible plants and determinants influencing their harvest, consumption, and conservation in south eastern Bhutan. *Plos one*, *18*(10), e0285936. https://doi.org/10.1371/journal.pone.0285936.

Bulya, T. E., Glukhareva, T. V., & Kovaleva, E. G. (2023). *Obtaining Cell Cultures of Medici-*

nal Plants In Recent Research and Advances in Soilless Culture. IntechOpen. https://doi.org/10.5772/intechopen.104650.

Davies, P.J. (2004). *Plant Hormones: Biosynthesis, Signal Transduction, Action* (Revised 3rdEd.). Kluwer Acad. Press.

Denton, O.A. (2014). *Crassocephalum crepidioides (Benth.) S. Moore.: Plant Ressources of Tropical Africa 2: Vegetables* (Grubben, G.J.H., Denton, O.A., Messiaen, C.-M., Schippers, R.R., Lemmens, R.H.M.J., Oyen, L.P.A., Ed.). Leiden.

Gopi C, & Vatsala T. M (2006). In vitro studies on effects of plant growth regulators on callus and suspension culture biomass yield from *Gymnema sylvestre. African Journal of Biotechnology, 5*(12), 1215-1219.

Hairuddin, R., Idris, M. Y., & Nur, K. (2023). Organogenesis of corn plants (*Zea mays* L.) at various concentrations o auxin and cytokinin plant growth regulators in vitro. *Asian Journal of Agriculture and Rural Development*, *13*(1), 91-97. https://doi.org/10.55493/5005.v13i1.4770.

Intanon, S., Wiengmoon, B. & Mallory-Smith, C. A. (2020). Seed morphology and allelopathy of invasive *Praxelisclematidea. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 48*(1), 261–272. https://doi.org/10.15835/nbha48111831

Kane, M. E. (2005). *Shoot Culture Procedure In: Plant Development and Biotechnology* (1st Ed), CRC Press.

Kaviani, B., Barandan, A., Tymoszuk, A., & Kulus, D. (2023). Optimization of in vitro propagation of pear (*Pyrus communis* L.) 'Pyrodwarf®(S)' rootstock. *Agronomy*, *13*(1), 268. https://doi.org/10.3390/agronomy13010268

Khan, A., Shah, A. H., & Ali, N. (2021). In-vitro propagation and phytochemical profiling of a highly medicinal and endemic plant species of the Himalayan region (*Saussurea costus*). *Scientific Reports*, *11*(1), 23575. https://doi.org/10.1038/s41598-021-03032-1

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Mohamad, M. E., Awad, A. A., Majrashi, A., Abd Esadek, O. A., El-Saadony, M. T., Saad, A. M., & Gendy, A. S. (2022). In vitro study on the effect of cytokines and auxins addition to growth medium on the micropropagation and rooting of Paulownia species (Paulownia hybrid and *Paulownia tomentosa*). *Saudi Journal of Biological Sciences*, *29*(3), 1598-1603. https://doi.org/10.1016/j.sjbs.2021.11.003

Mudau, F. N., Chimonyo, V. G. P., Modi, A. T., & Mabhaudhi, T. (2022). Neglected and underutilised crops: a systematic review of their potential as food and herbal medicinal crops in South Africa. *Frontiers in Pharmacology*, *12*, 809866. https://doi.org/10.3389/fphar.2021.809866

Nguyen, M. C. & Dang, T. P. T. (2020). Wound healing activity of *Crassocephalum crepidioides* (Benth.) S. Moore. Leaf hydroethanolic extract. *Oxidative medicine and cellular longevity, 2020*, 2483187. https://doi.org/10.1155/2020/2483187

Omonhinmin, C. A., Olomukoro, E. E., Onuselogu, C. C., Popoola, J. O., & Oyejide, S. O. (2023). rbcL gene dataset on intra-specific genetic variability and phylogenetic relationship of *Crassocephalum crepidioides* (Benth) S. Moore. (Asteraceae) in Nigeria. *Data in Brief*, *48*, 109266. https://doi.org/10.1016/j.dib.2023.109266

Oyebode, O. A., Erukainure, O. L., Sanni, O., & Islam, M. S. (2020). *Crassocephalum rubens* (Juss. Ex Jacq.) S. Mooreimproves pancreatic histology, insulin secretion, liver and kidney functions and ameliorates oxidative stress in fructose-streptozotocin induced type 2 diabetic rats. *Drug and Chemical Toxicology, 45*(2), 1–10. https://doi.org/10.1080/01480545.2020.1716783

Randall, R.P. (2017). *A Global Compendium of Weeds* (3rd Edition). Weeds Society of Western Australia.

Salami, T.B. (2019). Growth and fruit yields of thickhead (*Crassocephalum crepidioides* (Benth.) S. Moore) as influenced by propagule height and cutting surface preparation in Oyo, Nigeria. *Journal of Science, Technology, Mathematics and Education (JOSTMED), 15*(2), 178-184.

Schramm, S., Rozhon, W., Adedeji-Badmus, A. N., Liang, Y., Nayem, S., Winkelmann, T. & Poppenberger, B. (2021). The orphan crop *Crassocephalum crepidioides* accumulates the

pyrrolizidinealkaloid jacobine in response to nitrogenstarvation. *Frontiers in Plant Science*, *12*,702985. https://doi.org/10.3389/fpls.2021.702985

Sosnowski, J., Truba, M., & Vasileva, V. (2023). The impact of auxin and cytokinin on the growth and development of selected crops. *Agriculture*, *13*(3), 724. https://doi.org/10.3390/agriculture13030724

Vaishnav, D., & Chowdhury, P. (2023). *Plant Abiotic Stress Responses and Tolerance Mechanisms: Types and Function of Phytohormone and Their Role in Stress* (Ed. Saddam Hussain). IntechOpen. https://doi.org/10.5772/intechopen.109325

Xiong, Y., Chen, S., Wu, T., Wu, K., Li, Y., Zhang, X., & Ma, G. (2022). Shoot organogenesis and somatic embryogenesis from leaf and petiole explants of endangered Euryodendron excelsum. *Scientific Reports*, *12*(1), 20506. https://doi.org/10.1038/s41598-022-24744-y

Yuan, X. & Wen, B. (2018). Seed germination response to high temperature and water stress in three invasive Asteraceae weeds from Xishuangbanna, SW China. *PLoS ONE, 13*(1), e0191710. https://doi.org/10.1371/journal.pone.0191710.