JOURNAL OF
EMERGING INVESTIGATORS

Article

Genetic Bioaugmentation of Oryza sativa to Facilitate Self-Detoxification of Arsenic In-Situ

Prisha Bhat[1], Julie Baker[1]
[1] Plano East Senior High School, Plano, Texas

SUMMARY

Arsenic accumulation in rice, the world's popular grain crop, is becoming increasingly problematic because of the repeated use of arsenic-laden groundwater for cultivation. The World Health Organization reports that arsenic contamination of soil is a global issue, threatening the health of over 150 million people worldwide. Therefore, it is vital to prevent arsenic accumulation in the rice grain while simultaneously remediating arsenic from the soil. We hypothesized that if we genetically modified the rice plants using arsenic-resistant genes, then these rice plants would not only uptake arsenic from soil, but also be able to self-detoxify arsenic and thus be safe to consume. In order to test the hypothesis, we transformed rice cotyledons with *acr3* and *arsC* arsenic resistance genes using Agrobacterium tumefaciens. Transgenic rice plants were then grown in soil contaminated with 25 ppm arsenic. After four weeks, we observed a decline in soil arsenic from 25 ppm to 10 ppm. We also observed that the arsenic in the stem of transgenic rice plants to be less than one ppm compared to 6 ppm in non-transgenic rice plants, and arsenic was undetectable in the leaves of transgenic rice plants compared to 4 ppm in non-transgenic plants. We used the ANOVA test to assess the statistical significance. Therefore, phytoremediation, a technique using plants to clean environmental pollutants and utilizing rice plants transformed with arsenic-resistant genes, is a promising method for decontaminating polluted soil. It can significantly lower arsenic accumulation in the world's popular grain crop, thus potentially reducing serious health risks.

INTRODUCTION

Rice is the staple crop for more than half of the world's population, making it crucial to keep up with its production. Rice cultivation faces significant environmental challenges, especially with drought and arsenic contamination (1). Rice consumes nearly one-third of the world's freshwater resources for irrigation, as it is typically grown in flooded paddy fields to stave off weeds and pests and boost yields (1). However, groundwater is laden with several heavy metals, especially arsenic (1). Arsenic easily enters the environment through mining, smelting, and volcanic eruptions. Inorganic arsenate [As(V)] and arsenite [As(III)] are predominant in aquatic and soil environments (1). In the absence of rain, there is repeated use of arsenic-laden groundwater for rice cultivation (1). This has resulted in arsenic build-up in soil through the years, and is one of the main reasons for heavy arsenic contamination of rice (1) (2). Rice roots release oxygen, which causes oxidation of iron and leads to the formation of iron plaque at the rice root surface. The iron plaque facilitates the adsorption of arsenic by iron plaque, which then increases the rhizospheric concentration of arsenic around rice roots (2). In addition, arsenic contamination of soil is a known global public health concern. According to the EPA, soil arsenic levels ranging from 5 ppm to 20 ppm are generally considered safe (3).

Current heavy metal remediation techniques, including landfilling, vitrification, and electrokinetics, are expensive and potentially toxic, making them suboptimal (3). Phytoremediation, a known technique to naturally remediate heavy metals from the soil, only works with certain hyperaccumulator plants such as *Brassica juncea*. These plants are able to tolerate heavy metals as they have special transporters that sequester the heavy metals. However, these plants are not safe to consume if used for heavy metal remediation (4).

We sought to determine whether the reduction of arsenic in soil and rice grains can be simultaneously achieved if the rice plants are genetically modified using arsenic resistance genes that would facilitate self-detoxification of arsenic by the rice plants, compared to rice plants that are not genetically modified. This would not only reduce the soil arsenic content, but would also make the rice grain safe for consumption. Therefore, we first started analyzing the genes responsible for arsenic degradation. Based on the literature review and results obtained by data analysis from the National Center for Biotechnology Information (NCBI), we determined that *Oryza sativa* has several intrinsic genes that can potentially accumulate heavy metals, especially arsenic, but it also lacks some important genes that would facilitate degradation and arsenic extrusion from the plant cells (4). These genes are present and have been studied extensively in several microbes such as *Escherichia coli*, *Pseudomonas putida*, and *Burkholderia vietnamiensis* (5). Genetically modifying rice plants by transferring genes from microbes to enhance their ability to detoxify and expel arsenic can potentially eliminate arsenic accumulation in the rice grains and reduce arsenic levels in the soil. Amongst several genes that could successfully detoxify arsenic, we were able to obtain *acr3* (arsenical pump-driving ATPase) and *arsC* (arsenate reductase) genes. The *acr3* gene encodes for arsenic transporter which facilitates transport of As(III) out of the cells (6). The *arsC* gene encodes for arsenic reductase enzyme which reduces As(V) to As(III) (7). Although As(III) is the more toxic form of arsenic, it is also the most mobile form of arsenic, allowing it to exit the plant through phytovolatilization. In order to transfer the genes into the rice plants, we used

https://doi.org/10.59720/23-324

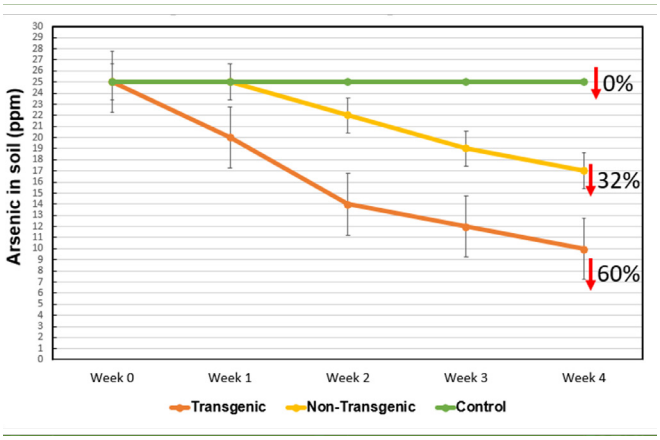

Figure 1: Soil arsenic levels at various stages of plant growth. Line graph showing mean ± SD soil arsenic levels (ppm) over four weeks. Genetically modified rice plants (n = 30) showed the greatest reduction in soil arsenic, with a 60% decrease by the end of four weeks. Negative control pot had no plants, and non-transgenic plants served as the positive control. One-way ANOVA, p = 0.0353.

the widely used *Agrobacterium tumefaciens* mediated genetic augmentation (8).

Based on this rationale, we sought to test the hypothesis that, when grown in arsenic-contaminated soil, *Oryza sativa* transformed with *arsC* and *acr3* genes will accumulate significantly less arsenic than the non-transformed plants. Furthermore, we hypothesized that we will observe a decrease in the levels of arsenic in the soil of transformed plants. At the end of the procedure, we observed a remarkable decline in the soil arsenic content and significantly less arsenic in the stem and the leaves of the transgenic plants. Therefore, genetically modifying rice plants with arsenic resistance genes from microbes can potentially reduce arsenic accumulation in rice grains and decrease soil arsenic levels. This approach addresses the problem of arsenic contamination in rice, making it safer for consumption and contributing to soil decontamination, which is essential for sustainable agriculture and public health.

RESULTS

To test the ability of transformed rice plants to self-detoxify and remediate arsenic from soil, we developed transgenic rice plants by transferring *arsC* and *acr3* genes using *agrobacterium tumefaciens*. We then conducted experiments using the transgenic rice plants and non-transformed rice plants in soil contaminated with 25 ppm arsenic. We then measured arsenic content in the soil, stem and leaves of all plant groups. After four weeks, we observed that there was a statistically significant difference in the soil arsenic content between transgenic rice plants and the negative control pot (p = 0.0353). Soil arsenic decreased from 25 ppm to 10 ppm, which was a 60% decline from baseline in pots containing transgenic rice plants, whereas the arsenic content remained unchanged in the negative control pot **(Figure 1)**. In the control with non-transformed rice plants, we also observed a statistically significant difference (p = 0.0424) in the soil arsenic content between non-transgenic rice plants and negative control pot, and the soil arsenic content decreased 32% from 25 ppm to 17 ppm **(Figure 1)**. Furthermore, we observed a statistically significant decline (p = 0.0405) in the arsenic content in the stem of the transgenic rice plants compared to the stem of the non-transgenic rice plants. Arsenic content in the stem of the transgenic rice plant was less than 1 ppm compared to 8 ppm in non-transgenic rice plants **(Figure 2)**. Finally, we observed a statistically significant decline (p = 0.0445) in the arsenic content in the leaves of the transgenic rice plants compared to the non-transgenic plants. Arsenic level in the leaves was almost undetectable in transgenic rice plants compared to 6 ppm in non-transgenic plants, indicating that the genetically modified rice plants were effective in self-detoxifying arsenic **(Figure 2)**.

We finally conducted a Spectrophotometric analysis to quantify the chlorophyll concentration in the dried leaves of all plant groups. We measured the optical density (OD) at specific wavelengths (chlorophyll a at 614 nm and Chlorophyll b at 435 nm). The OD was similar in both the control groups and experimental groups, thus indicating that the health of the genetically transformed rice plants after arsenic decontamination in the soil was comparable to the non-transformed plants **(Table 1)**.

DISCUSSION

The objective of our study was to determine whether genetically modifying rice plants with arsenic resistance genes from microbes could reduce arsenic accumulation in the rice grains and decrease arsenic levels in the soil. After four weeks of experimentation, we observed that the transgenic rice plants were 20% more effective in reducing soil arsenic than the non-transgenic plants. This difference in soil arsenic is likely due to increased arsenic uptake by the transgenic rice plants facilitated by the arsenic reductase enzyme. The *arsC* gene encodes the arsenic reductase enzyme, which reduces As(V) to As(III). As(III) is the more mobile form of arsenic, which also gets chelated by phytochelatins to get sequestrated in root vacuoles, thus preventing the heavy metal from entering the stem, leaves, and grain (**Figure 3**).

Furthermore, the arsenic content in the stem of the transgenic rice plants was significantly lesser than that of the non-transgenic plants. In addition, the arsenic in the leaves was almost undetectable in transgenic rice plants, while

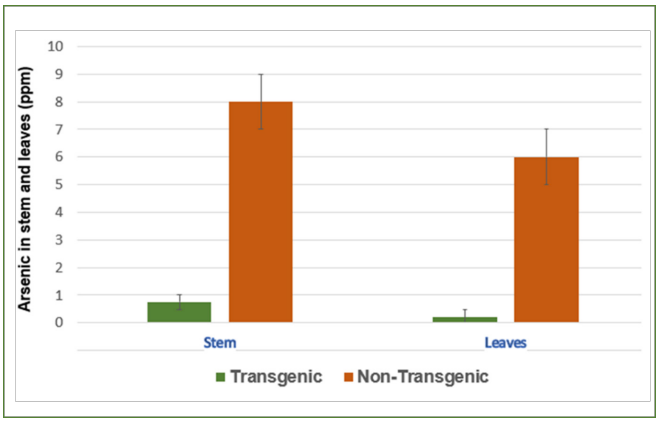

Figure 2: Arsenic level in the stem and leaves of the transgenic and non-transgenic plants at the end of four weeks. Bar graph showing mean ± SD amount of arsenic (in ppm) remaining in the stem and leaves at the end of four weeks (n = 30). Comparison between transgenic plants and non-transgenic plants (positive control). One-way ANOVA, p = 0.0405 (Stem), p = 0.0445 (Leaves).

https://doi.org/10.59720/23-324

Plant group	Chlorophyll a (OD)	Chlorophyll b (OD)
Transgenic	614 nm	435 nm
Non-Transgenic	614 nm	435 nm

Table 1: Spectrophotometric analysis for chlorophyll content in each plant group. Table showing absorbance values (OD) of chlorophyll a and chlorophyll b in leaves (n=5). Using a spectrophotometer, we measured optical density at 614 nm for chlorophyll a and 435 nm for chlorophyll b. Absorbance values for both experimental and positive control-chlorophyll plants grown in pots without arsenic were the same.

there was some remnant arsenic in the leaves of the non-transgenic plants. This finding is explained by the ability of the transgenic rice plants to self-detoxify arsenic facilitated by the Acr3 transporter. The Acr3 transporter is encoded by the *acr3* gene, which facilitates the extrusion of arsenic out of the leaves through the process of phytovolatilization (**Figure 4**). Although arsenic does escape into the environment, the amount extruded is negligible and gets diluted in the environment. If the arsenic content in the leaves is almost undetectable, then it can be concluded that the arsenic content in the rice grain is almost negligible and, therefore, safely consumed.

Some of the limitations in this experiment included the inability to determine the specific localization of the proteins expressed by the *arsC* and *arc3* genes within the rice plants. However, based on the results, these proteins are likely localized in the roots, stem, and leaves, as these are the primary sites of arsenic accumulation and detoxification. This inference is supported by the observed reduction in arsenic levels in both the soil and the various parts of the transgenic rice plants compared to the non-transgenic plants. Additionally, due to time constraints, we were unable to grow rice grains to measure arsenic content directly in the grains themselves. However, previous studies have shown a correlation between the nutrient content of leaves and grains, suggesting that the low arsenic levels observed in the leaves of transgenic rice plants may indicate similarly reduced arsenic levels in the grains (9 - 11). In the future we hope to expand this research by growing rice grains and measuring arsenic level in the rice grains. In addition, we hope to identify other genes that specifically localize in the root tissue that will contain the arsenic within the roots and thus prevent its entry into the plant and to the grain. We also hope to expand this research to other grain crops such as wheat, and to other heavy metals such as cadmium in the soil.

Results of the experiment supported our hypothesis, and in conclusion, our study successfully demonstrated that genetically modifying rice plants with arsenic-resistance genes from microbes significantly enhances their ability to reduce arsenic accumulation in soil and minimize its presence in rice grains. The transgenic rice plants exhibited greater efficacy in lowering soil arsenic levels compared to non-transgenic varieties, attributed to the enhanced arsenic uptake facilitated by the arsenic reductase enzyme and Acr3 transporter. This genetic modification also resulted in notably lower arsenic levels in the stems and leaves of the transgenic plants, suggesting minimal arsenic content in the grains, which is crucial for safe consumption. Although the study had limitations, including the inability to directly measure arsenic in the rice grains and the lack of precise protein localization, the results provide promising evidence for the potential of genetically modified rice plants to act as effective filters for environmental arsenic. Future research will focus on directly measuring arsenic levels in rice grains, identifying additional genes for more efficient arsenic sequestration, and expanding this approach to other crops and heavy metals. The advancements from this research can be translated into developing a sustainable, ecofriendly and cost-effective solution for eliminating environmental arsenic from the soil, increasing crop production and simultaneously developing self-detoxifying rice plants which are safe to consume.

MATERIALS AND METHODS

Computational Analysis/Gene Identification

We used publicly available data from The National Center for Biotechnology Information (NCBI) to assess various genes that have the potential to degrade arsenic. Of all the genes, we selected *acr3* and *arsC* genes after extensive literature review because preliminary studies were already conducted on these genes on arsenic-resistant Campylobacter isolates and using these genes as arsenic biosensors (6), (12). We obtained the genes from Dr. Barry Rosen's lab at the Florida International University.

Preparing 25 ppm arsenic solution

The arsenic solution was prepared using distilled water and 0.5% Sodium arsenite ($NaAsO_2$) obtained from RICCA chemical company. The mass of Sodium arsenite required was corrected by multiplying the mass of arsenic (desired amount) with (Molecular weight of $NaAsO_2$/ Atomic weight of As) while weighing $NaAsO_2$. To obtain 25 ppm of arsenic, 0.04 grams of Sodium arsenite dissolved in 1 liter of distilled water.

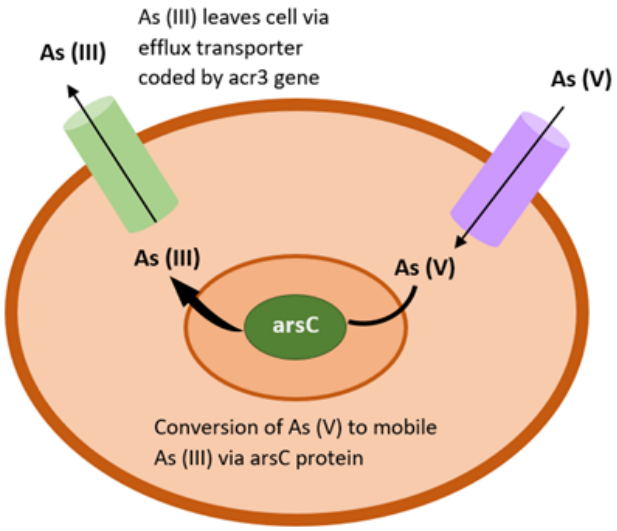

Figure 3: Roles of *arsC* and *acr3* genes in reducing arsenic in a cell. Snapshot of a cell demonstrating the mechanism of arsenic detoxification through genetically modified genes. The acr3 gene encodes for an arsenic transporter which facilitates the transport of As(III) out of the cells. The arsC gene encodes for an arsenic reductase enzyme which reduces As(V) to As(III). Although As(III) is the more toxic form of arsenic, it is also the most mobile form, allowing it to exit the plant through phytovolatilization.

https://doi.org/10.59720/23-324

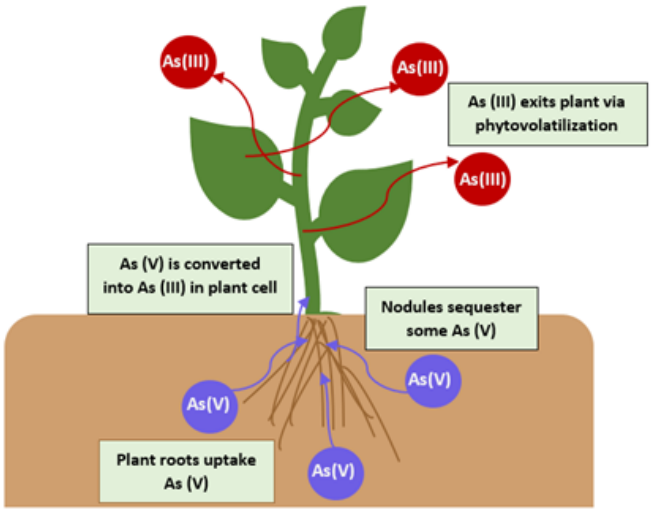

Figure 4: Potential mechanism for transgenic rice plant self-detoxifying arsenic. A potential mechanism for transgenic rice plant self-detoxifying arsenic. Figure demonstrating the pathway involved in arsenic detoxification by the genetically modified rice plant. As(V) is taken up by the roots and the shoots are converted to As(III). Some of As(III) is sequestered in the root nodules, and the remaining exits the plant by the process of phytovolatilization. Although As(III) is the more toxic form of arsenic, it is also the most mobile form, allowing it to exit the plant through phytovolatilization.

Testing the arsenic content using the standard protocol in the Arsenic QuickTM Kit

Commercially available potting soil was used as the substrate for all experiments according to manufacturer's instructions (Scotts Miracle Gro, Marysville, Ohio, U.S.).
32 oz clear plastic jars were used as pots for the plants and soil. One tablespoon of soil from the pot was taken and left to air dry overnight. 0.5 g of the dried soil was transferred to the reaction bottle supplied in the Arsenic Quick™ Kit, and then mixed with distilled water and the reagents provided in the testing kit. After 15 minutes, the color change in the test strip was compared with the color chart that was provided in the arsenic testing kit by the manufacturer, which provided various arsenic levels based on the color in the test strip.

Construction of Plasmids and Transformation of *Agrobacterium*

We obtained *arsC* and *acr3* genes were from Dr. Rosen's lab at the Florida International University. We collaborated with Gold Biotechnology company to transform *Agrobacterium tumefaciens* with these genes. The genes were ligated into one plasmid. *Agrobacterium* was transformed to include the two genes of interest, using the freeze-thaw technique, where we mixed the *A. tumefaciens* and 1 ml of the genetic material, froze it in liquid nitrogen (-80 degree Celsius) for ten seconds, and then placed it in 37-degree Celsius water bath for 5 minutes. seconds. We confirmed the transformation and arsenic resistance by incubating the transformed *A. tumefaciens* in Luria Broth (LB) broth along with sodium arsenate, verifying color change to yellow with silver nitrate reagent.

Plant Transformation

We obtained *Oryza sativa L* (IR64 rice cultivar – non-GMO), from the International Rice Research Institute. We germinated 100 paddy seeds by soaking them in moisture for four days until the cotyledons were visible. We wounded sixty cotyledons by making 1 mm incisions using a sterile scalpel and incubated them with the transformed *Agrobacterium* in LB broth at room temperature for three days, and then transferred them to the co-culture Murashige Skoog (MS) plant tissue for incubation for five days in dark under a sterile hood with lights turned off at room temperature. When the callus became visible, we transferred all cotyledons with callus into MS resting media with kanamycin to get rid of the *A. tumefaciens*. After three days, we transferred the cotyledons with the callus into MS regeneration media and incubated for ten days at room temperature to facilitate both root and shoot formation. Unwounded forty cotyledons were simultaneously grown in the regeneration media at room temperature to serve as positive control.

Plant Growth/Pot experiments

Once we successfully grew the transgenic plants in the regeneration media, we then transferred the ten transgenic plants to each of the three pots labeled 'Transgenic Rice' (experimental group), and non-transformed plants into each of the three pots labeled 'Non-Transgenic Rice' (positive control). We also transferred ten non-transformed plants to one pot with no arsenic in soil to only assess plant health (positive control-chlorophyll - to only assess chlorophyll content at the end of the experiment). We also left a pot with arsenic contaminated soil as it is (negative control) without any plants. The pots were placed in a small greenhouse outside the lab. All pots received the same amount of sunlight and humidity was regulated within the greenhouse. We watered all pots each day including the negative control pots without the plant, with 300 ml of distilled water. We measured soil arsenic level using Arsenic Quick™ each week for four weeks in all pots, except the positive control chlorophyll pot. After four weeks, we also measured arsenic level in the stem and the leaves in both experimental and positive control pots. Finally, we analyzed the chlorophyll content of the leaves in all experimental and positive control-chlorophyll pots.

Analyzing the chlorophyll content of the leaves

Five 5" dried leaves from each experimental and 'positive control-chlorophyll' pots were obtained and placed in a plastic bag. Thirty mL of isopropyl alcohol was poured into each bag, and leaves were crushed gently to obtain green liquid extract, which was filtered using a coffee filter. Leaf extract was poured into plastic cuvettes. The spectrophotometer was calibrated using isopropyl alcohol and the optical density of each test group was analyzed to determine the chlorophyll a and b content. The excitation wavelength was 614 nm for chlorophyll a and 435 nm for chlorophyll b for all plant groups.

Statistical Analysis

We conducted a one-way analysis of variance (ANOVA) test to examine the effects of transgenic rice plants on the arsenic level in the soil and the rice plants' stems and leaves. The independent variables in the analysis included the three groups (transgenic rice, non-transgenic rice, and control), while the dependent variable was the arsenic content measured in ppm. Tukey's Honestly Significant Difference (HSD) post-hoc test was further used to determine if there was

https://doi.org/10.59720/23-324

a significant difference in the results between the individual groups.

ACKNOWLEDGMENTS

We would like to sincerely thank Dr. Barry Rosen, distinguished university professor at the Florida International University and his team members Dr. Masafumi Yoshinaga and Dr. Duan who not only graciously donated the plasmid samples, but also guided us through the process of developing a transgenic plant. Without their help, we would not have been able to conduct my experiment, as the special arsenic resistant genes were not available anywhere in the US. We would like to express our gratitude to Dr. Abhaya Dandekar, distinguished professor and director of the plant transformation facility at UC Davis, who guided us on the underlying mechanisms involved in plant transformation.

Received: November 24, 2023
Accepted: June 29, 2024
Published: December 03, 2024

REFERENCES

1. Muehe, E. M., et al. "Rice Production Threatened by Coupled Stresses of Climate and Soil Arsenic." Nature Communications, vol. 10, no. 1, 2019. https://doi.org/10.1038/s41467-019-12946-4
2. Satyapal, G. K., and N. Kumar. "Arsenic: Source, Distribution, Toxicity and Bioremediation." Arsenic Toxicity: Challenges and Solutions, 2021, pp. 153-163. https://doi.org/10.1007/978-981-33-6068-6_6
3. Selvi, Adikesavan. "Integrated Remediation Processes Toward Heavy Metal Removal/Recovery From Various Environments." Frontiers in Environmental Science, vol. 7, 22 May 2019. https://doi.org/10.3389/fenvs.2019.00066
4. Sayers, E. W., et al. "Database Resources of the National Center for Biotechnology Information." Nucleic Acids Research, vol. 50, no. D1, 07 Jan. 2022, pp. D20-D26. https://doi.org/10.1093/nar/gkab1112
5. Cai, L., et al. "Genes Involved in Arsenic Transformation and Resistance Associated with Different Levels of Arsenic-Contaminated Soils." BMC Microbiology, vol. 9, no. 1, 2009. https://doi.org/10.1186/1471-2180-9-4
6. Chen, Jian, and Barry P. Rosen. "Biosensors for Inorganic and Organic Arsenicals." Biosensors, vol. 4, no. 4, 2014, pp. 494–512. https://doi.org/10.3390/bios4040494
7. Garbinski, L. D., et al. "Pathways of Arsenic Uptake and Efflux." Environment International, vol. 126, 2019, pp. 585-597, https://doi.org/10.1016/j.envint.2019.02.058
8. Hooykaas, P. J. "Transformation of Plant Cells via Agrobacterium." Plant Molecular Biology, vol. 13, no. 3, Sep 1989, pp. 327-336. https://doi.org/10.1007/BF00025321
9. Silva, D., Dias, L. A. S., and F. Matos. "Correlation between Leaf Nutrient Contents and Grain, Oil and Protein Productivities in Jatropha curcas L." Revista Facultad Nacional de Agronomía Medellín, vol. 76, no. 3, 2023, pp. 10457-10464. https://doi.org/10.15446/rfnam.v76n3.105714
10. Fischer, S., Hilger, T., Piepho, H.-P., Jordan, I., & Cadisch, G. (2021). Missing association between nutrient concentrations in leaves and edible parts of food crops – A neglected food security issue. Food Chemistry, 345, 128723. https://doi.org/10.1016/j.foodchem.2020.128723
11. Hou, W., Tränkner, M., Lu, J., Yan, J., Huang, S., Ren, T., Cong, R., & Li, X. (2020). Diagnosis of nitrogen nutrition in rice leaves influenced by potassium levels. Frontiers in Plant Science, 11, 165. https://doi.org/10.3389/fpls.2020.00165
12. Wang, L., et al. "Identification of an Arsenic Resistance and Arsenic-Sensing System in Campylobacter jejuni." Applied and Environmental Microbiology, vol. 75, no. 15, 2009, pp. 5064-5073. https://doi.org/10.1128/AEM.00149-09

Copyright: © 2024 Bhat and Baker. All JEI articles are distributed under the attribution non-commercial, no derivative license (http://creativecommons.org/licenses/by-nc-nd/4.0/). This means that anyone is free to share, copy and distribute an unaltered article for non-commercial purposes provided the original author and source is credited.