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Agronomic and Genetic Biofortification of Rice Grains with Microelements to Assure Human Nutritional Security

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Abstract

'Hidden hunger' or micronutrient deficiency due to malnutrition among underprivileged and economically-challenged people, including pre-school children and women, has emerged as a major health-related issue, mostly in the developing nations like Africa, South Asia and Latin America. Globally, micronutrient malnutrition alone affects more than two billion people, mostly among resource-poor families in developing countries, with Zn and Fe deficiencies, being the most prevalent. Approximately, five million children die out of micronutrient deficiency every year. Rice (*Oryza sativa* L.), the major staple food, contributes up to 50-80% of daily calories for more than half of the global population. White milled (polished) rice, preferred for human consumption, suffers from micronutrient loss, so that biofortification of rice grains would be a sustainable and cost-effective approach for people who primarily consume rice and have limited access to diverse food items. Among the biofortification strategies being discussed as major solution to microelement deficiency, agronomic biofortification as foliar spray or soil broadcasting with chemical fertilizers or nanoparticles, and genetic biofortification via molecular breeding or genetic engineering have proved to be effective in enhancing the level of microelements in rice grains. This review focuses on the progress in rice grain biofortification with microelements like Zn, Fe, Se and B and future prospects of biofortified rice in alleviating 'hidden hunger' in humans.

Keywords: Agronomic supplementation; Genetic biofortification; Hidden hunger; Zinc; Iron; Selenium; Boron; Rice

Introduction

The concept of 'hidden hunger' has emerged in the last two decades amongst two billion people worldwide as a result of inadequate intake of key micronutrients, despite intake of major calories through the staple food crops. A carbohydrate-rich diet including rice, wheat or maize (the major staple food) may satisfy hunger, but 'hidden hunger' can only be mitigated when the diet has enough of essential micronutrients [1]. Micronutrient deficiency is a silent epidemic condition, for the underprivileged people of the world, since it slowly cripples the immune system, stunts physical and intellectual growth and even causes fatal death. Such condition remains hidden or unnoticed for a long period of time till they surface out through some diagnostic symptoms, often too late to be reversed. More than 24,000 people die daily owing to malnutrition and 'hidden hunger' globally. The common and traditional approaches to alleviate human micronutrient deficiency are food fortification, providing varied dietary sources and healthy food, and medical supplementation. However, for the large proportion of poor rural residents from the developing countries with limited resources and lower income, any of these strategies is highly expensive or unaffordable. In the developing and less developed countries, providing access to a more diverse and balanced diet that can ameliorate the micronutrient deficiency is a sheer challenge. This problem can be resolved through enhancement of bio-available micronutrients in the edible parts of food crops, popularly known as biofortification, which is advantageous for people experiencing difficulty in changing their dietary habits due to financial, cultural and religious restrictions, as well as for the Government of a nation, since it is sustainable and inexpensive as compared to nutrition supplement program [2]. Another definition of the problem is nutrigenomics which is described as manipulating plant micronutrients to improve human health by the interface of plant biochemistry, genomics and human nutrition [3].

Rice (*Oryza sativa* L.) is the dominant staple food for more than half of the world's population. Rice provides 21% of energy and 15% of protein requirements of global human populations. It provides 50-80% of the daily caloric requirement for more than three billion people, but it does

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not provide enough essential microelements, especially zinc (Zn) and iron (Fe), to eliminate Zn and Fe deficiency. Hulling of field-harvested paddy (rough rice) produces brown rice, the most nutritious form of processed rice. Unpolished brown rice contains important minerals like Zn, Fe, Cu, Ca, P and several vitamins. However, rice consumers prefer polished (milled) white rice because they are soft, light, easy to digest and faster to cook. During the polishing process, the bran layer along with subaleurone, embryo and a small part of the endosperm are lost, and the micronutrients which are mostly present in these layers are also drained away, so that the nutritive values are highly lowered, though these reductions vary with rice genotypes and milling process. Although brown rice consumption has increased with the spread of education and awareness, a vast proportion of consumers still prefer polished rice, which has made endosperm-specific, nutrient biofortification so essential for white rice [4]. Agronomic approaches, conventional plant breeding, or genetic strategies have been adopted as one of the measures to enhance the content of several microelements like Zn, Fe, Se and B in rice, the staple food, for their daily intake by the rural residents, thereby alleviating their deficiency. These biofortification strategies will be discussed in the underlying sections.

Zinc Biofortification

Zinc (Zn) deficiency in humans persists in almost 27-30% of global population and is associated with severe health complications like retarded physical growth in children, defective immune system, hypogonadism, DNA damage, cancer, increased incidence of infection, disturbed learning abilities, mental lethargy, alopecia, acrodermatitis and infertility [5]. World Health Organization has ranked Zn deficiency as the 6th among top 10 major causes of illness in developing countries. Children are more prone to Zn deficiency which globally causes about 4.4% of the total child death, with half a million children under five years of age dying every year. Zn requirements of malnourished children are estimated to be between 2-4 mg kg^{-1} body weight [6], which is higher than those for healthy children (0.17 mg kg^{-1}) at 1-3 years. The polished rice contains on an average only 12 mg kg^{-1} Zn, whereas the recommended dietary intake of Zn is 12-15 mg per day [7]. In most cases, rice-cultivated soils are very low in plant-accessible Zn, leading to 80% decrease in grain Zn concentration, which escalated Zn deficiency problem by reducing its bioavailability [8]. Wissuwa et al. (2008) [9] reported native soil Zn status to be the dominant factor than genotype and fertilizer in determining grain Zn concentrations. Several sources of Zn like $ZnSO_4$ (21-26% Zn), $ZnCl_2$, ZnO, $Zn(NO_3)_2$, Zn-EDTA, Zn oxy-sulfate and Zn-coated urea could be used as fertilizers, of which $ZnSO_4$ is the most common [10]. Alternate wet and dry cycle in paddy field combined with $ZnSO_4$ rather than Zn-EDTA fertilization was demonstrated as an effective method to elevate grain yield and increase Zn accumulation in rice grains [11]. Foliar application, particularly with $ZnSO_4$ has been suggested to be more efficient in grain Zn accumulation over soil application [12,13]. Jaksomsak et al. (2018) [14] also found that Zn foliar spray significantly increased Zn accumulation in unpolished rice in all the varieties, ranging from 17% to 50% increase. Phattarakul et al. (2012) [15] showed that a foliar Zn spray applied at late growth stage to rice grown under field conditions caused a greater increase in grain Zn, than a foliar Zn spray before flowering stage. Pandey et al. (2013) [16] and Boonchuay et al. (2013) [17] recommended that foliar application should be done at the later stages, i.e., around or after the flowering period for increased Zn content in the grains. Wu et al. (2010) [18] found that higher translocation of Zn from flag leaves to grains occurred when Zn was applied at booting or anthesis stage; foliar application of Zn (0.5% $ZnSO_4$) in this case was done at the time of panicle initiation. Similar findings by Mabesa et al. (2013) [19] also highlighted foliar Zn application during early milk stage to be most effective in increasing grain Zn concentration. One of the reasons for the stimulated transport of Zn into seeds after the flowering stage could be related to significant increases in protein biosynthesis during the early stage of seed formation. Increasing seed protein concentrations creates a sink for Zn, so that there is a close positive correlation between seed protein and Zn concentrations [20]. Gomez-Coronado et al. (2016) [21] observed that combined foliar and soil application of Zn-enriched fertilizers at the right rate, time, and stage is effective in grain Zn intake. Kumar et al. (2016) [22] in his experiments found that when zinc was applied through combined soil and foliar mode, there was increase of grain Zn concentration by 36%. Zn supply through soil (basal) together with foliar spray during (maximum tillering + flowering) stage caused a significant increase in Zn concentrations in cooked rice grain [23]. Similar observation was also made by Cakmak (2010) [24] and Barua and Saikia (2018) [25]. However, Farooq et al. (2018) [26] found somewhat different result which reported that all of the Zn application methods, *viz*., soil application, foliar spray, seed priming, and seed coating increased Zn concentration in grains of aromatic rice grown in dry seeded and puddle transplanted production systems. In another work by Kheyri et al. (2019) [27], application of Zn, either as Zn-nanoparticles (300 g ha^{-1}) or to soil (9 g ha^{-1}) to the indica rice variety, Tarom Hashemi improved grain Zn concentration. A global Zn fertilizer project called HarvestZinc project (www.harvestzinc.org) under HarvestPlus program has been set up, whose purpose is to evaluate the potential of Zn-containing fertilizers for increasing the zinc concentration in cereal grains and improving crop production in different target countries like India, Pakistan, China, Thailand, Turkey, Mozambique, Zimbabwe and Brazil. The uptake, allocation and accumulation of Zn is also regulated by Yellow Stripe-Like (YSL) proteins such as ZIP1, ZIP3 and ZIP4 which helps Zn-phytosiderophore complex formation, along with other genes like *HMAs* [28], *MTPs* [29], *NASs* [30], *YSLs* [31], *ZIPs* [32], and *ZIF1* [33], which are all involved in the biosynthesis of phytosiderophores. Guerinot (2000) [34] stated that ZRT/IRT-like proteins and ZIP like transporters were important for Zn uptake into the roots. Engineering rice with the mentioned genes, either singly or in combination, can promote enhanced Zn accumulation in the grains. Zn concentration in rice grains was increased when *OsYSL2* was over expressed under *OsSUT1* promoter [35]. Transgenic rice overepressing rice heavy metal *ATPase 2 (OsHMA2)* under the same promoter accumulated slightly more Zn in the seeds [36].

Iron Biofortification

Iron (Fe)-Deficiency Anemia (IDA) is one of the most prevalent human micronutrient deficiencies in the world, affecting an estimated one-third of the world's population and causing 0.8 million annual deaths worldwide, the risk being higher in South Asian countries. The people most vulnerable to IDA are women and children. IDA can hamper cognitive and physical development, reduce immunity, and enhance the risk of maternal and perinatal mortality. The polished rice contains on an average only 2 mg kg^{-1} Fe, whereas the recommended dietary intake of Fe is 10-15 mg per day [7]. Rough rice contains about 38 ppm of Fe which is reduced to 8.8 ppm in brown rice after processing and 4.1 ppm in milled rice [37]. Masuda et al. (2009) [38] suggested that Fe content of 19 ppm in brown rice was reduced almost five times to around 4 ppm in polished grains. The breeding target to fulfill the 30% Estimated Average Requirement (EAR) for women and children recommended by the HarvestPlus program for Fe is 13 mg g^{-1} in polished rice or around five to six fold increase of grain Fe in popular rice. These figures indicate the need of Fe biofortification in milled rice. Fe accumulation in rice grains is regulated by several processes like uptake by the roots, root to shoot transfer, ability of leaf to load Fe in the phloem, which in turn delivers Fe to the developing grains via phloem sap [39]. Significant enrichment in grain Fe content up to 80% was reported via foliar application of Fe-containing solutions like Fe-amino acid and $FeSO_4.7H_2O$ [40]. Kumar et al. (2016) [22] also reported that foliar application at 0.5% increased the Fe concentration in the grain. Even nitrogenous fertilizers enhanced Fe concentration in the polished grains of the rice variety, Zhenong 952 (Zhang et al. 2008) [41] indicating that nitrogen management by high dose of nitrogen application represents a promising agronomic strategy to improve micronutrient contents in cereal grains [22]. Jin et al. (2008) [42] observed that combined foliar application of boron (B) and Fe-amino acid complex ($FeSO_4.7H_2O$ bound to amino acids) to japonica rice variety, Bing 98110 increased the Fe (3.0 mg kg^{-1}) and Zn content in polished rice by 19% and 27% respectively, along with other nutritive values like proteins and 16 amino acids. Fakharzadeh et al. (2020) [43] have recently applied 2.5 g L^{-1} of nano-chelated Fe fertilizer at nursery and booting stages, and observed increase in nitrogen, phosphorus, potassium, iron and zinc concentrations in white rice of T3 generation by 46%, 43%, 41%, 25% and 50%, respectively, in addition to protein content, showing that biofortification in rice is possible even without genetic modification.

Conventional plant breeding has developed the semi-dwarf rice variety, IR68144 by crossing between IR8 and Taichung (Native)-1. This variety produces 21 μg g^{-1} of Fe in brown rice and retains about 80% of its Fe concentration even after polishing [44]. Several strategies of genetic modification for Fe biofortification have been undertaken from time to time by different research groups, given that Fe translocation and Fe homeostasis in rice have begun to be understood at the molecular level. The first approach was enhancement of Fe accumulation in rice seeds by *ferritin* gene expression under the control of endosperm-specific promoter. Ferritin is a ubiquitous protein for Fe storage and stores about 4,000 Fe atoms in a complex. The soybean *ferritin* gene, *SoyferH1* was expressed in rice driven by the endosperm-specific rice promoter, *glutelin* (*GluB1*) by Goto et al. (1999) [45] and Qu et al. (2005) [46] which increased the seed Fe content. The second approach involved over expression of barley *Nicotinamine Synthase* (NAS) gene, *HvNAS1* which increased Fe concentration three-fold in the polished rice grains. Nicotinamine (NA), synthesized by NAS enzyme, chelates metal cations like Fe(II) and Zn(II) and helps in the internal transport of Fe [38]. In the third approach, the rice *OsYSL2* was over expressed in the panicle and immature seeds during the seed maturation stage under rice *sucrose transporter* promoter, *OsSUT1*, which increased Fe concentration in polished rice seeds by up to three-fold. The OsYSL2 is the rice NA-Fe(II) transporter protein participating in internal Fe transport [35]. Introduction of *mugineic acid synthase* gene, *IDS3* from barley in rice enhanced Fe concentration in polished rice seed up to 1.25 and 1.4 times in calcareous and normal soil cultivation in field respectively. Mugineic Acid (MA) family phytosiderophores are natural Fe(III) chelators, used in Fe acquisition from the rhizosphere. Rice produces deoxymugineic acid (DMA) which facilitates uptake and internal transport of Fe, whereas barley biosynthesizes not only DMA, but also MA by MA synthase, IDS3 [38]. The rice phenolics efflux transporters, phenolics efflux zero 1 and 2 (PEZ1 and PEZ2), secrete phenolics in to apoplasm (in roots and in xylem) to solubilize apoplasmic Fe for transport [47]. Rice lines that over express iron-regulated transporter-like protein 1 (OsIRT1) accumulated more Fe and Zn in the seeds [48]. The knockdown of *OsVIT* genes in rice increases Fe by 1.4 fold as well as Zn in grains and decreases them in flag leaves. The rice vacuolar iron transporter genes (*OsVIT1* and *OsVIT2*) are involved in the transport of Zn and Fe into vacuoles *via* tonoplast [49]. Kobayashi et al. (2013) [50] showed that RNA interference (RNAi)-mediated silencing of *OsHRZ2* in rice led to 3.8-fold more Fe in brown rice and 2.9-fold more Fe in polished rice grain. The hemoerythrin motif-containing Really Interesting New Gene (RING) and zinc finger protein 1 (OSHRZ1) and OsHRZ2 ubiquitin ligases bind with Fe and Zn and possess ubiquitination activity. Over expression of OsIRO2 resulted in 2.0-fold higher amounts of Fe in brown rice grains of transgenic rice than control rice. The basic helix loop helix transcription factor, OsIRO2 acts as a positive regulator of Fe deficiency responses in rice [51]. Another strategy adopted was to lower the phytic acid level in rice grains via RNAi-mediated silencing of *IPK1* gene [that encodes the final step key enzyme, inositol-1, 3, 4, 5, 6-pentakisphosphate 2-kinase (IPK1) of phytic acid metabolism], using *oleosin 18* (*Ole18*) seed-specific promoter. Phytic acid accumulates as mixed salts called phytate, having six negatively charged ions, making it a potent chelator of divalent cations such as Fe, Zn, Ca and Mg thereby reducing their bioavailability [52]. However, none of these transgenic approaches appeared full-proof, since the amount of Fe that is actually required to mitigate IDA in humans could not be met by the transgenic polished rice seeds generated by the above engineering methods. The target Fe content of over 15 ppm in polished seeds of field-grown rice has not been fulfilled by such approaches. Therefore, the current focus is to adopt gene pyramiding approach, i.e., combination of multiple transgenes for Fe homeostasis. Masuda et al. (2012) [53] combined three transgenic approaches where simultaneously Fe storage in grains was increased by over expressing *ferritin* under endosperm-specific promoter, Fe translocation was enhanced by over expressing *NAS* under *actin1* promoter, and Fe flux into endosperm enhanced *via* over expression of *OsYSL2* under the control of an endosperm-specific promoter and *sucrose transporter* promoter. The paddy field-grown T3 polished seeds exhibited 4.4-fold higher Fe content than that in non-transgenic seeds (0.9 μg g^{-1}), along with 1.6 times increased Zn content, with no defect in yield. The promising results for Fe-enriched rice grains in tropical indica rice were developed by Trijatmiko et al. (2016) [54] and Wu et al. (2019) [55] by expressing the endosperm storage gene *PvFER*, the chelator *AtNAS1* gene and an intracellular iron store, *AtNRAMP3* in one cassette. Wu et al. (2019) [55] achieved 13.65 mg g^{-1} Fe level in the greenhouse condition, while Trijatmiko et al. (2016) [54] reported 15 mg g^{-1} Fe in polished grains, together with enhanced Zn content. A combination of four genes (*AtIRT1, Pvferritin, AtNAS1* and *Afphytase*) has been transferred in the rice variety, Taipei-309, resulting in 4.3-fold Fe increase in polished grain; *Afphytase* encodes *Aspergillus flavus* phytase enzyme that hydrolyzes phytic acid releasing the chelated minerals and phosphate, increasing their bioavailability [56].

Selenium Biofortification

Selenium (Se) deficiency affects about 15% of world population, with infants being at higher risk. Globally, almost 1.0-1.5 billion people suffer from Se deficiency and therefore suffer from several diseases like some forms of cancer, hypothyroidism, hampered immune functions, cognitive decline, male infertility and cardiovascular disorders [57]. It is recommended that a human individual should intake 55-70 μg Se daily, which may exceed to 300 μg per day for lowering the incidence of cancer [58]. Selenite and selenate are the two water-soluble chemical forms which can be absorbed by the plants after foliar application. The efficiency of this process depends on rice genotypes, growing season, edaphoclimatic conditions and technological designs of application. Soil rich in Fe and Al oxides have the tendency to retain anions like selenate, reducing its bioavailability. Plants cultivated in soils with low Se contents (<0.5 mg kg^{-1}) are unable to accumulate this micronutrient in adequate amounts for human health. Hence, Se must be added to the soil or incorporated into fertilizers that are commonly broadcasted in the field. This can increase Se level both in soil as well as in edible grains. Wang et al. (2013) [59] showed that spraying rice with sodium selenite (10.5 g Se ha^{-1}) increased grain Se contents up to 51-fold (from 0.03 μg g^{-1} to 1.54 μg g^{-1}). Zayed et al. (1998) [60] also reported that the accumulation of Se in rice through foliar application with selenite was higher than that with selenate. Chen et al. (2002) [61] pointed out that Se concentration in rice increased after foliar fertilization with sodium selenite; such increase being 36% higher when using sodium selenate. Hu et al. (2002) [62] also reported a 10-fold increase in Se contents of rice grains after a single foliar spraying (14-18 g Se ha^{-1}) with sodium selenate at the heading stage of rice. Lidon et al. (2018) [58] showed that foliar fertilization with sodium selenite and sodium selenate in the range of 120-300 g Se ha^{-1} led to a high accumulation of Se in the grains of four rice varieties, Ariete, Albatros, OP1105 and OP1109, with Albatros and OP1105 genotypes showing better response, and sodium selenite being more efficient. This also led to increase in total lipids, mostly oleic, linoleic and palmitic acids in all the genotypes, along with several sugars like sucrose, glucose, raffinose and fructose, and proteins. de Lima Lessa et al. (2019) [63] showed that soil application of 47 and 36 g ha^{-1} of Se (as sodium selenate) may guarantee the production of rice grains with adequate Se levels, fit for human consumption, without affecting grain yield. Foliar Se application promoted higher Se accumulation in the Mozambican rice cultivar, IR-87684-23-2-3-2 [64]. Se biofortification of rice sprouts also appears to be a feasible way to promote Se and phenolic acid intake in human diet, with well-known health benefits [65]. Farooq et al. (2019) [66] proposed that 10 mg L^{-1} sodium selenite could be recommended as appropriate for foliar fertilization in the organic selenium biofortification of Se-free rice, since most organic selenium (0.03 mg kg^{-1}) was accumulated in polished rice. Premarathna et al. (2012) [67] showed that selenate-enriched urea granules applied by broadcast method to floodwater at heading stage increased grain Se concentrations 5-6-fold (by 450-600 μg kg^{-1}) compared to the control (no fertilizer Se applied). The organic Se species, selenomethionine and selenomethylcysteine are rapidly loaded into the phloem and transported to the grain far more efficiently than inorganic species. Organic Se species are distributed more readily and extensively throughout the grain (external grain layer, and endosperm) than selenite, which is retained at the point of grain entry [68]. Such a thorough understanding of accumulation and distribution of Se species within the rice grains is required for efficient Se biofortification programs.

Boron Biofortification

Field experiments across multiple locations in rice-growing areas of Punjab and Pakistan with low boron (B) calcareous soils (0.21-0.42 mg B kg^{-1}) and low organic matter (0.8-1.8%) revealed B deficiency as a major nutritional problem [69,70]. Rice grains suffer from B deficiency as the plants are grown on high pH and alkaline soils with low B contents, low soil organic matter, and inadequate use of B fertilizer, which restricts the availability, uptake and accumulation of B into grains. Due to non-availability of cost-effective, B-enriched fertilizers, farmers normally do not prefer to apply B fertilizers which further aggravate the deficiency. The different sources of B fertilizers may be boric acid, borax, sodium tetraborate and Solubor (either solid or solution), as well as crushed minerals like ulexite, datolite and colemanite. Rashid et al. (2007) [70] observed that application of 0.75 kg B ha^{-1} *via* broadcasting method in transplanted rice, growing on calcareous soil, could overcome B deficiency, where only 1.7-3.4% (plant basis) of the applied B was taken up by the rice plants. Small amounts of B fertilizers need to be mixed with major nutrient fertilizers. Boron can also be applied using alternative methods like foliar application and the dipping of rice seedlings in B solution. However, the utilization efficiency of B by these methods varies with the age of the crop, rate of B used, and soil and environmental factors. Transgenic rice lines over expressing different B transporters help in efficient uptake of B by plant roots and xylem loading. The xylem loading efflux transporter, BOR1 participates in active transport system in loading B into the xylem, while channel protein NIP5;1, expressed in root epidermis, is involved in facilitated diffusion and symplastic movement. Transfer of such genes, using efficient molecular approach could assist in the transfer of B-efficient trait in rice [71]. Moreover, polyols are important metabolites responsible for retranslocating B within the plants. Therefore, engineered rice for higher polyol synthesis will also lead to better manipulation of internal B translocation, resulting in rice plants with high B concentration [72].

Conclusion

Despite numerous efforts to tackle mineral deficiencies through either agronomic supplementation or biotechnological fortification, deficiencies prevail among two billion people. Malnutrition due to microelement deficiency in diet is a common problem, particularly in developing countries, because of intake of monotonous diets, solely consisting of staple food crop, rice, which provide the daily calorie intake. Hence, rice biofortification is supposed to play an important role in alleviating the burden of poor or low nutrition. Agronomic fertilization is not considered as a long-term sustainable approach in developing countries, because some fertilizers are costly and dangerous to the environment, causing the accumulation of toxic by-products in soil, which results in deterioration of soil ecological environment and increases the incidence of genotoxicity and health hazards. The success of genetic biofortification is again greatly limited by the fact that there is a world-wide regulatory slowdown in the overall approval of transgenic crops, following all the field-trials and biosafety guidelines, so that they have practically undergone total market failure without contributing any benefit to the consumers, particularly the hungry population who need such products the most. Most of the developing countries do not have necessary infrastructure and sophisticated laboratories for analysis of safety issues and food quality, adequate marketing strategies, proper policies and political will. Therefore, the crop scientists need to address this problem through some alternate technologies. Nanochelating technology could be a novel approach in this regard, but again, the appropriate size and concentration of nanoparticles, together with the specific time of their applications to the field-grown rice plants need to be standardized and their ecotoxicity determined. As a result, there is an urgent need to find a safe and efficient way to increase the quality and quantity of agricultural products. Probably, adoption of any such isolated approach may not be effective in the present scenario to keep pace with the alarming situation, and it remains to be seen if a combined strategy of integrating foliar supplementation, breeding program, genetic fortification and nanoparticle application fulfils in achieving the target microelement level in the polished rice grains. In a nutshell, rice fortification with microelements should top the priority list of research and technological advancement studies, particularly in the worst-affected nations like Africa, South Asia and Latin America, as a real nexus in improving human health *via* nutritional security.

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