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Selenate-Enriched Urea Granules Are a Highly Effective Fertilizer for Selenium Biofortification of Paddy Rice Grain

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Supporting Information

ABSTRACT: This study examined the effects of applied selenium (Se) species, time of application, method of application, and soil water management regimen on the accumulation of Se in rice plants. Plants were grown to maturity in a temperature- and humidity-controlled growth chamber using three water management methods: field capacity (FC), submerged until harvest, and submerged and drained 2 weeks before harvest. Two Se species, selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), were applied at a rate equivalent to 30 g ha^{-1}. Four application methods were employed as follows: (i) Se applied at soil preparation, (ii) Se-enriched urea granules applied to floodwater at heading; (iii) foliar Se applied at heading; and (iv) fluid fertilizer Se applied to soil or floodwater at heading. Total Se concentrations in rice grains, husks, leaves, culms, and roots were measured, as well as Se speciation in grains from the Se-enriched urea granule treatment. Highest Se concentrations in the grain occurred with SeO_4^{2-} and with fertilizer applied at heading stage; SeO_4^{2-}-enriched urea granules applied at heading increased grain Se concentrations 5−6-fold (by 450−600 μg kg^{-1}) compared to the control (no fertilizer Se applied) in all water treatments. Under paddy conditions other Se fertilization strategies were much less effective. Drainage before harvesting caused Se to accumulate in/on rice roots, possibly through adsorption onto iron plaque on roots. Rice grains contained Se mainly in the organic form as selenomethionine (SeM), which comprised >90% of the total grain Se in treatments fertilized with SeO_4^{2-}-enriched urea granules. The results of this study clearly show that of the fertilizer strategies tested biofortification of Se in rice grains can best be achieved in lowland rice by broadcast application of SeO_4^{2-}-enriched urea granules to floodwater at heading stage.

KEYWORDS: *selenium, selenite, selenate, biofortification, fertilizer, rice, Se-enriched urea*

INTRODUCTION

Selenium is an essential micronutrient for humans and animals.[1,2] Toxicity and deficiency of Se in humans and animals are separated by a very narrow margin compared to other nutrients.[3] Cancers may be induced by oxidative-related conditions. Selenium can activate antioxidant enzymes in the human body.[1] Antioxidants are compounds that block the action of free radicals that can damage cells and DNAs. Different strategies have been tested or implemented worldwide to achieve optimum Se concentrations in humans;[3] the most common strategies include the consumption of high-Se foods (e.g., Brazil nuts), individual supplementation, Se supplementation to livestock,[4] and biofortification of food crops.[4−6]

Biofortification is the increase in the bioavailable concentration of elements in edible portions of crop plants through either fertilization (agronomic biofortification) or crop selection and breeding (genetic biofortification).[4−6] Agronomic biofortification is an easy and cheap method to increase Se concentrations in edible portions of crops.[7] Selenium fertilizer programs have successfully been implemented in Finland for forage and cereal crops, and increased Se concentrations in animal and human populations have been achieved.[5] In Finland the Se intake per capita has increased from 25 to 124 μg day^{-1}.[5] Increasing the Se concentration in foods such as wheat and rice is an appropriate target to increase human Se intake because they are staples for most of the world's population. Selenium fertilizer programs have been developed for wheat, but no effective Se program has yet been developed for rice.

Rice can be grown in upland or lowland conditions, and different fertilization strategies may be needed for each scenario, in terms of both Se species used and method of fertilizer application (foliar or applied to soil). The availability of soil applications of Se is often much greater under upland conditions compared to flooded soils,[8,9] but the majority of rice crops around the world are lowland cultivated.[10] In terms of the most effective Se species, for foliar applications SeO_4^{2-} has

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been found to be more effective than SeO_3^{2-} for Se biofortification of rice.[6] There are no data to evaluate the effectiveness of soil-applied SeO_3^{2-} or SeO_4^{2-} for biofortification of rice, but these species appear to be equally available to wheat plants under aerobic conditions in solution culture.[1] However, in aerobic soils the addition of SeO_4^{2-} often leads to greater accumulation of Se in plants than does the addition of SeO_3^{2-},[11,12] likely due to greater retention of SeO_3^{2-} by soils.[11,13,14] Developing a Se fertilization program for lowland rice is challenging because soil-applied Se availability depends on redox and pH conditions in submerged soils. The availability to rice of soil-applied SeO_4^{2-} fertilizer in flooded soils has been found to be low,[9,15] due to either reduction to SeO_3^{2-}, which is more strongly retained by soils than SeO_4^{2-},[13,16] or reduction of Se oxyanions to elemental Se(0) in anoxic soils.[17] Hence, foliar Se fertilization of crops is often preferred[18] and has been tested on rice.[19,20] However, the disadvantages of foliar fertilization are the additional labor involved in separately applying Se from other broadcast granule applied nutrients, as well as the lack of any residual effect for subsequent crops. An effective alternative fertilization strategy for Se where macronutrients (nitrogen, phosphorus, or potassium) are applied simultaneously with Se would therefore be advantageous.

Previously we showed that the potential availability of soil-applied SeO_4^{2-}, SeO_3^{2-}, and Se (0) differed markedly in both aerobic and anaerobic soils.[21] Application of Se fertilizers in any form during soil preparation was ineffective because Se availability (as determined by isotopic dilution) decreased rapidly after soil flooding.[22] Preharvest oxidation of paddy soil is likely to release little available Se due to the slow oxidation of Se(0) to SeO_3^{2-} and also the slow oxidation of SeO_3^{2-} to SeO_4^{2-}.[23] Hence, a more effective Se fertilization strategy is required.

Bioavailability of Se for humans and animals largely depends on the species of Se consumed rather than the total Se concentration. Organic Se species in the diet are more bioavailable than inorganic Se species.[24,25] Organic Se compounds such as methylselenocysteine (MeSeCys), selenomethionine (SeM), and γ-glutamyl-Se-methylselenocysteine (γ-glutamyl-MeSeCys) are effective chemoprotective agents, which may prevent the development of breast, liver, and prostate cancers,[24,26] but there is a paucity of studies on the organic and inorganic Se species present in rice grains following application of different Se fertilizers to plants or soils.

Here we report experiments to elucidate the effect of Se species, time of application, soil water regimen, and four Se application methods on the yield of rice and the accumulation of Se in grains, culms, leaves, and roots under both upland and paddy growth conditions. The speciation of Se in grains from the SeO_4^{2-}-enriched urea granule treatment was further undertaken to examine the effect of increased Se accumulation on Se species present in grains.

■ MATERIALS AND METHODS

Standards and Reagents. All reagents and standards used were of trace metal grade, and ultrapure deionized water (Milli-Q, Millipore) was used for all chemical preparations and dilutions. Sodium salts of sodium selenite and sodium selenate, SeM, and citric acid were purchased from Sigma (Australia). Individual stock solution of SeO_3^{2-} at 1000 mg Se L^{-1} was purchased from SPEX-Certiprep, USA. Selenomethionine selenoxide (SeOM) was prepared through the addition of excess hydrogen peroxide (0.1 mL of 30% H_2O_2) to 2 mL of SeM (100 mg of Se L^{-1}).[27]

Pot Experiment 1. Plants were grown in 3.5 L black plastic pots lined with plastic bags and filled with 2 kg of the Hanwood loam (a Rodoxeralf[37]) collected near Griffith, a rice-growing area of Australia. The soil had a pH of 6.36 (1:5 soil/water suspension),[28] EC = 141.2 $\mu S\ cm^{-1}$,[28] total carbon of 2%,[29] cation exchange capacity (CEC) of 21 cmol(+) kg^{-1}, and a total Se concentration of 0.117 mg kg^{-1}. All pots received the equivalent of 150 kg N ha^{-1} (half applied at soil preparation and half at the heading stage), 25 kg P ha^{-1}, and 100 kg K ha^{-1}, together with a micronutrient mix of 0.3 $ZnSO_4$, 0.3 $CuSO_4$, 0.1 H_3BO_3, 20 $CaSO_4$, 20 $MgSO_4$, and 0.01 $(NH_4)_6Mo_7O_2 \cdot 4H_2O$ kg ha^{-1}[30] applied during soil preparation. Soils were either submerged or maintained at field capacity (FC) for 14 days before two 18-day-old healthy rice seedlings (*Oryza sativa* sp. Amaroo) were transplanted. Two weeks before harvest, submerged treatments were drained, and the moisture content was thereafter maintained at FC. Plants were grown until final harvest at physiological maturity.

Treatments and Experiment Design. The experiment was conducted in a light-, temperature-, and humidity-controlled growth chamber. Maximum and minimum temperatures inside the growth chamber were 30 and 25 °C, a 12 h daylight cycle was used, and high humidity (53%) was maintained. There were 81 pots in a randomized complete block design with a factorial arrangement of the treatments: two Se species, that is, SeO_3^{2-} and SeO_4^{2-}; three water treatments, that is, field capacity, submerged, and submerged then drained; and four application methods (see below).

Application of Selenium Fertilizer. The rate of Se application was equivalent to 30 g ha^{-1} based on application rates used in previous studies[31] and assumptions regarding loss of Se by sorption to soil. Selenite and SeO_4^{2-} fertilizers were added to soil either at soil preparation or heading stage. Selenium applied as either SeO_4^{2-} or SeO_3^{2-} at soil preparation was sprayed as a dilute solution of sodium selenite or sodium selenate diluted in ultrapure deionized water (Milli-Q, Millipore) onto the soil and mixed thoroughly. At the heading stage either SeO_4^{2-} or SeO_3^{2-} was applied by three methods; SeO_4^{2-}, SeO_3^{2-}-enriched urea granules, or fluid Se fertilizer was applied to the soil surface. The Se-enriched urea was prepared by spraying Se (sodium selenite or sodium selenate in high-purity deionized water solution) onto the urea granules and allowing the granules to dry at 30 °C in an oven. The fluid Se fertilizer was a solution of either sodium selenite or sodium selenate in high-purity deionized water. Foliar SeO_4^{2-} or SeO_3^{2-} fertilizer was either sodium selenite or sodium selenate in high-purity deionized water and was sprayed carefully onto leaves using aerosol sprayers at heading stage. Pots receiving the foliar fertilizer at heading were separated from other treatments to avoid contamination during spraying.

Rice Sample Preparation for Analysis. At maturity, plants were harvested and shoots, roots, and grains were separated. Roots were cleaned using reverse osmosis (RO) water, 1% sodium lauryl sulfate ($CH_3(CH_2)_{10}CH_2OSO_3Na$) (Sigma), and finally high-purity deionized water. Plant samples (grain, leaf, culm, and root) were dried at 55 °C to a constant weight and dry weight (DW) recorded (grain). Husks were removed from harvested grains using a laboratory-scale hand-operated dehulling machine. The plant tissues were ground using a laboratory seed grinder and sieved through a sieve with a diameter of 500 μm.

Total Se Analysis. The grain samples were digested using a closed-vessel microwave procedure (Ethos E touch control, Milestone, North America) using a two-stage time program: 5 min at 300 W and 40 min at 500 W. Approximately 0.5 g of finely ground grain samples was weighed into a Teflon digestion vessel and 10 mL of concentrated HNO_3 acid (Aristar) added. After microwave digestion, the vessels were allowed to cool for 30 min at room temperature, and then the contents were diluted to 50 mL with ultrapure deionized water (Milli-Q, Millipore). Digest solutions were filtered through a 0.22 μm filter (Sartorius) and analyzed for total Se concentrations by inductively coupled plasma−mass spectrometry (ICP-MS) (Agilent 7500ce ICP-MS with H_2 gas added to the collision cell at a flow rate of 4 mL min^{-1}). The accuracy of the digestion and ICP-MS analysis procedure was assessed through the analysis of certified reference materials NIST 1568a rice flour and NIST 1573a tomato leaves. The total Se concentrations determined in the rice and tomato certified reference material were in close agreement with the certified value (NIST 1568a, this study, 0.38 ± 0.04 mg Se kg^{-1} ($n = 3$), certified value = 0.38 ± 0.04 mg Se kg^{-1}; NIST 1573a, this study, 0.054 ± 0.003 mg Se kg^{-1} ($n = 3$), certified value = 0.054 ± 0.006).

Enzymatic Extraction of Selenium Species for Chromatographic Speciation. Approximately 0.2 g of ground grain tissue from the SeO_4^{2-}-enriched urea granule treatments was weighed into 15 mL Pyrex culture tubes with 20 mg of protease XIV (Sigma) and 6 mL of ultrapure deionized water (Milli-Q, Millipore). The samples were shaken end-over-end at 37 °C in an incubator for 24 h, centrifuged at 1200*g*, and filtered through a 0.22 μm filter. The resulting solutions were analyzed for Se species (OSeM, SeO_4^{2-}, SeM, and SeO_3^{2-}) by high-performance liquid chromatography−inductively coupled plasma−mass spectrometry (HPLC-ICP-MS).[32] The operating conditions for HPLC-ICP-MS are summarized in Table 1. The identification of Se species occurred through retention time comparisons with synthetic standards, and concentrations were determined using peak areas.

Table 1. Operating Conditions for HPLC-ICP-MS

isocratic chromatographic parameters	
column	Hamilton PRP-X100 anion exchange column (Phenomenex) (250 × 4.6 mm, 10 μm)
mobile phase	10 mM citric acid buffer 2% (v/v) methanol
pH	5.5; pH was adjusted using NH_3 solution
column temperature (°C)	25
flow rate (mL min^{-1})	1.0
injection volume (μL)	50
ICP-MS parameters (Agilent 7500ce)	
isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se, and ^{82}Se
total analysis time (s)	900

Pot Experiment 2. A second experiment was performed to examine uptake of Se from SeO_4^{2-}-enriched urea granules and pure selenourea (CH_4N_2Se). Environmental and soil conditions were identical to those outlined above for pot experiment 1, and treatments consisted of either SeO_4^{2-}-enriched urea granules, SeO_4^{2-}-enriched urea−ammonium nitrate (UAN), or pure seleno-urea. At rice heading stage, Se fertilizers were applied onto floodwater in a manner similar to that of the first experiment.

Floodwater samples were collected from 1−2 cm above the soil−water interface at 1 and 10 days after Se fertilizer application. At each sampling time, 10 mL of floodwater was collected between two rice plants and filtered into centrifuge tubes using 0.22 μm filters (Millipore millex-GS). The floodwater solutions were acidified with 50 μL of 6 M HCl and analyzed for total Se concentration using ICP-MS.

Plant sap sample collection was undertaken following the method used by Li et al.[33] Shoots were cut from 3 cm above the water level. The cut surface was washed using ultrapure deionized water and blot dried with clean tissues before sap samples were collected for 2 h. Total Se concentrations in sap samples were determined using ICP-MS.

Statistical Analysis. The significance of fertilizer-applied Se species, application time, application method, and soil water management on grain dry yield (grain) and Se concentrations in rice tissues (grain, leaves, culm, and roots) were determined using analysis of variance (ANOVA) in Genstat software (Genstat 10th ed., VSN International, Hempstead, U.K.). Least significant differences (LSD) were used for comparison of the treatment means.

■ RESULTS AND DISCUSSION

Treatment Effects on Grain Yield. Rice plants growing in aerobic soil (FC) had lower grain dry weights than the other water treatments ($p \leq 0.05$) with little or no effect of Se species or application method (Supporting Information, Table 1). The reason for higher DW in grains from submerged and submerged/drained treatments could be the availability of some nutrients in FC soils being lower than that in submerged soils (Supporting Information, Table 2). We did not measure nutrients in floodwater for this study; however, as other studies have reported elsewhere, stabilization of the pH around neutrality in submerged rice soils has some implications for the availability of nutrients.[34] In addition, the ability of the rice plant to grow under aerobic conditions is often less.[22] In particular, soil solution concentrations of P, and K, normally increase with submergence.[10] These are essential nutrients for root development and tillering,[10] which ultimately determine final yield. Method of Se application had little consistent effect

Table 2. Effect of Applied Se Species, Method of Application, and Water Management on Grain and Husk Se Concentrations ($n = 3$)[a]

		Se concentration[a] (mg kg^{-1})					
		grain			husk		
moisture treatment	Se application method	SeO_3^{2-}	SeO_4^{2-}	control	SeO_3^{2-}	SeO_4^{2-}	control
FC	at soil preparation	0.059 a	0.079 abcd	0.076 abc	0.025 a	0.029 ab	0.030 abc
	Se-enriched urea at heading	0.063 ab	0.405 lm		0.030 abc	0.230 kl	
	foliar at heading	0.273 kl	0.150 hij		0.157 k	0.050 defg	
	fluid at heading	0.094 bcdefg	0.407 m		0.055 defgh	0.208 kl	
submerged	at soil preparation	0.085 abcde	0.092 bcdef	0.086 abcde	0.091 j	0.056 defghi	0.036 abcd
	Se-enriched urea at heading	0.117 defghi	0.590 m		0.077 ghij	0.299 l	
	foliar at heading	0.122 efghi	0.105 cdefgh		0.090 lmno	0.084 hij	
	fluid at heading	0.089 abcde	0.166 ij		0.046 cdef	0.071 fghij	
submerged/drained	at soil preparation	0.082 abcde	0.092 bcdef	0.097 cdefg	0.046 cdef	0.046 cdef	0.043 bcde
	Se-enriched urea at heading	0.138 ghij	0.485 m		0.063 efghij	0.295 l	
	foliar at heading	0.136 fghij	0.176 ij		0.068 fghij	0.080 hij	
	fluid at heading	0.109 cdefgh	0.189 jk		0.056 defghi	0.086 ij	

[a] Different letters in the table for grain and husk Se concentrations separately are significantly different for the three-way interaction of Se application method × applied Se species × water management.

Table 3. ANOVA Table for the Statistical Analysis of Grain Se Concentrations

source of variance	DF	SS	MS	vr	*F* prob
Se application method (A)	4	2.17033	0.54258	46.16	<0.001
water management (B)	2	0.03245	0.01622	1.38	0.260
applied Se species (C)	2	1.27325	0.63663	54.17	<0.001
A × B	8	0.53626	0.06703	5.70	<0.001
A × C	2	1.53315	0.76658	65.22	<0.001
B × C	4	0.03292	0.00823	0.70	0.595
A × B × C	4	0.27580	0.06895	5.87	<0.001
residual	54	0.63468	0.01175		
total	80	6.48885			

Table 4. Effect of Applied Se Species, Method of Application, and Water Management on Leaf and Culm Se Concentrations ($n = 3$)

		Se concentration[a] (mg kg^{-1})					
		leaf			culm		
water treatment	Se application method	SeO_3^{2-}	SeO_4^{2-}	control	SeO_3^{2-}	SeO_4^{2-}	control
FC	at soil preparation	0.043 a	0.045 a	0.046 a	0.046 abc	0.042 ab	0.060 bcdef
	Se-enriched urea at heading	0.053 ab	0.269 gh		0.032 a	0.200 klmn	
	foliar at heading	0.427 hi	0.458 i		0.216 lmno	0.170 jklm	
	fluid at heading	0.065 ab	0.313 ghi		0.052 abcde	0.235 lmnop	
submerged	at soil preparation	0.106 cde	0.113 cde	0.077 bc	0.046 abcd	0.105 ghij	0.080 defgh
	Se-enriched urea at heading	0.084 bcd	0.351 hi		0.076 cdefg	0.289 nop	
	foliar at heading	0.393 hi	0.318 ghi		0.391 p	0.252 mnop	
	fluid at heading	0.110 cde	0.129 de		0.150 ghij	0.092 fghi	
submerged/drained	at soil preparation	0.125 de	0.136 ef	0.157 ef	0.105 ghij	0.120 ghij	0.109 ghij
	Se-enriched urea at heading	0.150 ef	0.379 hi		0.145 ijkl	0.240 lmnop	
	foliar at heading	0.327 ghi	0.294 ghi		0.221 klmno	0.248 mnop	
	fluid at heading	0.153 ef	0.206 fg		0.132 hijk	0.137 ijk	

[a] Different letters in the table for leaf and culm and concentrations separately are significantly different for the three way interaction of Se application method × applied Se species × water management.

on grain yields, except in FC treatments where the control was significantly lower than Se fertilizer treatments.

Treatment Effects on Se Accumulation in Rice Plants.

Grain and Husk Selenium Accumulation. Concentrations of Se in rice grains and husks under different treatments are shown in Table 2. The largest part of the variance in Se concentrations was explained by application method, Se species, and their interaction (Table 3). Overall, SeO_4^{2-} treatments led to highest Se concentrations in grains, whereas SeO_3^{2-} treatments led to the lowest grain concentrations. However, the three-way interaction of Se application method × applied Se species × water treatment was significant for both grain and husk Se concentrations ($p \leq 0.001$). Selenium applied at the heading stage led to higher grain Se than Se applied at soil preparation. Perhaps, at heading stage, plants are more physiologically active and mobilizing nutrients to fill the grains with photosynthetic products more rapidly. Also, by heading stage, plant roots were well developed and well distributed, ready for nutrient uptake. Furthermore, Se applied at soil preparation had 2 weeks without plants and, by the time plants were introduced into the pots, Se added as SeO_3^{2-} would have been sorbed onto/into soil colloids/minerals and SeO_4^{2-} may have been reduced.[22] By the time the root system of transplanted rice was ready for nutrient uptake, most of the added Se may have been converted to unavailable forms such as selenide (Se^{2-}) or elemental selenium (Se(0)).

The highest grain Se concentration was recorded for SeO_4^{2-}-enriched urea granules applied at heading stage for all water treatments. In FC soils, fluid SeO_4^{2-} applied at heading also had a statistically similar effect on accumulating Se in grains. Higher grain Se concentrations in the fluid SeO_4^{2-} treatment applied at heading in FC treatments were expected because SeO_4^{2-} has been shown to be highly available in aerobic soils.[35−38] In addition, studies on upland crops such as wheat and barley have also recorded higher grain Se concentrations with soil-applied SeO_4^{2-} than with SeO_3^{2-}.[4,39]

When Se was applied to soil at planting, drainage of floodwater before harvest had no effect on increasing Se concentrations in grain, suggesting that oxidation of any reduced Se species in soil to SeO_4^{2-} was too slow to influence crop Se accumulation.

Foliar application at the heading stage gave the highest grain Se concentration (0.27 mg kg^{-1}) for those plants that received SeO_3^{2-} in FC soils. In a study conducted in China examining foliar application of Se in paddy rice, researchers reported Se concentrations of 0.355 and 0.411 mg kg^{-1} for two different varieties given SeO_3^{2-} at a rate of 18 g ha^{-1}.[20] In another study, a rate of 20 g ha^{-1} of SeO_3^{2-} led to a grain Se concentration of 0.471 mg kg^{-1}.[19] However, in our study, grain Se concentration in submerged pots with foliar SeO_3^{2-} was far below those values (Table 2). In another submerged field trial, testing for SeO_3^{2-} foliar spray showed that to achieve 40−75 $\mu g\ kg^{-1}$ Se in

Table 5. Effect of Applied Se Species, Method of Application, and Water Management on Root Se Concentrations ($n = 3$)

		root Se concentration[a] (mg kg^{-1})		
water management	Se application method	SeO_3^{2-}	SeO_4^{2-}	control
FC	at soil preparation	0.208 ab	0.143 a	0.173 a
	Se-enriched urea at heading	0.193 a	0.429 def	
	foliar at heading	0.163 a	0.175 a	
	fluid at heading	0.356 cde	0.416 cdef	
submerged	at soil preparation	0.441 ef	0.387 cdef	0.311 bc
	Se-enriched urea at heading	0.496 f	0.499 f	
	foliar at heading	0.317 bcd	0.371 cde	
	fluid at heading	0.432 ef	0.428 def	
submerged/drained	at soil preparation	0.780 gh	0.768 gh	0.219 ab
	Se-enriched urea at heading	1.008 j	0.863 hi	
	foliar at heading	0.678 g	0.812 h	
	fluid at heading	0.940 ij	0.853 hi	

[a] Different letters in the table for root concentrations are significantly different for the three-way interaction of the Se application method × applied Se species × water management treatment.

rice grains, the solution should contain 20−30 μg Se L^{-1}.[40] These values cannot be compared with our data because there was no information given about how much solution was sprayed per plant or area and also no information on whether they considered the Se concentrations in control pots not receiving Se fertilizer. Possible reasons for the different Se values among different studies could be varietal differences[7] and time remaining until harvest after fertilizer application. For instance, in the study undertaken by Hu et al.,[20] plants had ∼3 months after fertilizer application at heading until harvest, at which stage the plants had a long time to transfer Se from leaf to grains. In our study, the elapsed time was a little over 1 month from heading to harvest.

Selenium accumulation in husks was generally lower than that of grain. Selenate-enriched urea granules and fluid SeO_4^{2-} applied at heading had similar husk Se concentrations in aerobic soils, but SeO_4^{2-}-enriched urea granule treatment was much more effective than all other treatments in accumulating Se in rice husks in submerged and submerged/drained treatments (Table 2). Overall, SeO_4^{-2}-enriched fertilizers were more effective than SeO_3^{2-}-enriched fertilizers in increasing husk Se concentrations.

Selenium Accumulation in Leaves and Culms. The three-way interaction among applied Se species, application time, and method of application was statistically significant ($p \leq 0.001$). However, interaction effects between Se application methods × applied Se species had significant and major effects on leaf Se concentration ($p \leq 0.001$). Highest leaf Se concentrations were for foliar-applied Se in all soils (Table 4), likely due to retention of foliarly applied Se to leaf surfaces. As observed for grain Se data, SeO_4^{2-}-enriched urea granule treatments also had high Se concentrations in leaves and culms, and in this case the Se must have derived from root uptake.

Selenium Accumulation in/on Roots. By far the greatest proportion of the variation in Se concentrations in roots was explained by water management method, with FC soils having the lowest root Se concentrations and submerged/drained soils the highest (Table 5). Even though the roots were thoroughly cleaned before analysis, we cannot be certain whether this accumulation occurred inside the roots or on the root surface, as we observed iron plaque on the roots of submerged plants. Iron plaque is known to strongly sorb oxyanions,[41,42] and it is therefore highly likely that SeO_3^{2-} and/or SeO_4^{2-} sorbed to the iron plaque.

Selenium Application Method. Application of fertilizer Se to soil prior to planting was ineffective, possibly due to reactions of Se in submerged soils that reduced the availability of both SeO_3^{2-} and SeO_4^{2-}.[21] Application of Se at heading to the floodwater appears to be the most effective biofortification strategy, and SeO_4^{2-} was generally more effective than SeO_3^{2-} in this regard. There was no consistent advantage of foliar Se over fluid Se fertilizer applied to the floodwater. However, it is evident from the results that the application of SeO_4^{2-}-enriched urea granules to floodwater at heading is a very promising way to deliver Se to paddy rice to stimulate Se biofortification of grains. There are several possible mechanisms to explain the efficiency of this treatment. The presence of N fertilizer (urea, mostly in the form of NH_4^+ in rice soils) may have influenced absorption of Se from the root mat at the soil surface, as after application to floodwater the Se-coated urea granules sank onto the exposed rot mat at the interface of the soil and floodwater. Plant roots exposed to high NH_4^+ or urea have the potential to absorb more anions.[43,44] Alternatively, there may be more efficient translocation of Se inside the plant when Se is applied with urea. Efficient translocation of Fe in the presence of N fertilizer in wheat plants has been shown previously.[45] Higher leaf Se concentrations in the submerged treatments receiving Se-enriched urea granules, compared to those receiving fluid Se, lend support to the idea of efficient translocation of Se when Se is coapplied/colocated with urea. It should be noted, however, that the fluid Se treatments also received urea, although in this case the Se and urea were not colocated. A third hypothesis was that Se applied with urea may have reacted to form selenourea (CH_4N_2Se) in the granules or at the soil/floodwater interface, and this enhanced the absorption of Se by the roots. Very little is known of the formation and reactions of selenourea in soils. Sorption of selenourea to iron hydroxides is much less than that of selenite,[46] and it is known that selenourea forms in reduced environments.[47] However, it is readily oxidized,[48] and we believe it is unlikely to be stable in rice floodwaters or in the oxidized rhizosphere of rice roots. We tested the hypothesis that selenourea could be taken up by rice plants in experiment 2, in which pure selenourea was applied to floodwaters and persistence in floodwater determined, as well as translocation to the xylem of rice plants growing under submerged conditions. Whereas the addition of selenourea resulted in higher concentrations of Se in floodwater (compared to SeO_4^{2-}-enriched UAN and urea) 1 day after application, it did not persist and Se was not detectable in floodwater 10 days after fertilizer application (Table 6). The concentration of Se in rice xylem sap was highest with selenourea 1 day after fertilizer application, but 9 days later Se concentrations in rice xylem sap were highest with SeO_4^{2-}-enriched urea granule treatment (Table 5).

Table 6. Selenium Concentration in Floodwater and Plant Xylem Sap Collected at Two Times during the Second Experiment

selenium treatment	sample location	1 day after Se application	10 days after Se application
Se-enriched urea, 10 μg kg^{-1}	floodwater	0.33 ± 0.05	<0.20
pure selenourea, 10 μg kg^{-1}		1.47 ± 0.05	<0.20
UAN[a] + Se, 10 μg kg^{-1}		0.60 ± 0.16	<0.20
control		<0.20	<0.20
Se-enriched urea, 10 μg kg^{-1}	xylem sap	2.00 ± 0.0	8.40 ± 0.85
pure selenourea, 10 μg kg^{-1}		3.33 ± 1.09	5.00 ± 1.41
UAN + Se, 10 μg kg^{-1}		2.00 ± 0.0	<0.2
control		<0.20	<0.2

[a] UAN, urea ammonium nitrate solution.

Selenium Speciation in Rice Grain. In this study, the SeO_4^{2-}-enriched urea treatment had significantly higher concentrations of Se in grains and husks than the other treatments. Therefore, rice grain samples from this treatment were used for speciation studies by HPLC-ICP-MS. Enzymatic hydrolysis using Protease XIV extracted 93 ± 7% of the total Se present in the grain samples. The results of the speciation analysis showed that SeM was the predominant species in grains of the SeO_4^{2-}-enriched urea treatment (Figure 1). Quantitative data from the HPLC-ICP-MS analysis (column recovery ~ 90−100%) indicate that SeM comprised >90% and SeOM ~ 9% of the total extracted grain Se. Similar results have been reported elsewhere.[49] Our results clearly show that applied inorganic Se (SeO_4^{2-}) accumulates in the rice grains as organic Se in the form of SeM, which is more bioavailable for humans than inorganic Se species.[9] The SeO_4^{2-}-enriched urea treatment did not cause the accumulation of different Se species than those expected with a foliar or fluid SeO_4^{2-} fertilizer.

In summary, differences in Se accumulation in rice plants were a function of the species of applied Se, time of application, method of application, and soil moisture regimen. More Se accumulated in rice plants when SeO_4^{2-} was the Se species used in fertilizer in all water regimens. This study confirmed previous findings that Se application preplanting is not an effective method of enhancing accumulation of Se in rice plants. Heading was the best time for Se application. For both aerobic and submerged rice pots, Se fertilizer applied at the heading stage, as SeO_4^{2-}-enriched urea, was extremely effective as an agronomic biofortification strategy. Selenium accumulation in rice plants decreased from rice roots in the order grains > leaves > culms and husks. Drainage preharvest caused increased Se accumulation in/on rice roots, possibly through adsorption onto iron plaque. Selenium in the rice grains accumulated from application of SeO_4^{2-}-enriched urea was mainly in the form of SeM, which is highly bioavailable. Coating or incorporation of SeO_4^{2-} onto urea is simple and inexpensive, and as farmers often apply a side dressing of urea to floodwaters during crop growth, the practice is a simple and extremely effective way to supply Se to crops and to biofortify grains with bioavailable Se. Further studies are needed to confirm the effectiveness of this fertilizer strategy under field conditions and to understand the mechanisms responsible for the enhancement of Se uptake observed with this fertilizer combination.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

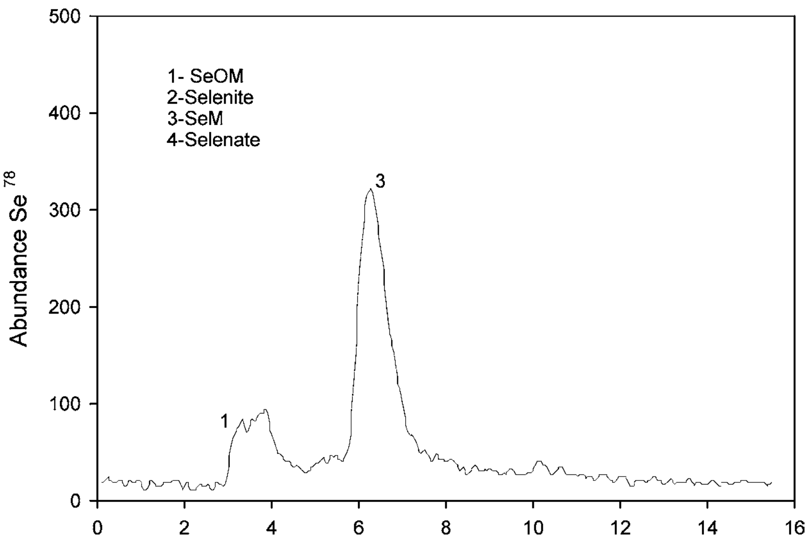

Figure 1. Selenium speciation in protease-extracted rice grains of plants growing in pots treated with SeO_4^{2-}-enriched urea granules applied at heading.

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■ ABBREVIATIONS USED

MeSeCys, methylselenocysteine; SeM, selenomethionine; γ-glutamyl-MeSeCys, γ-glutamyl-Se-methylselenocysteine; SeOM, selenomethionine selenoxide; CEC, cation exchange capacity; FC, field capacity; RO, reverse osmosis; DW, dry weight; ICP-MS, inductively coupled plasma-mass spectrometry; HPLC, high-performance liquid chromatography; UAN, urea-ammonium nitrate; ANOVA, analysis of variance; LSD, least significant differences.

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