Comparing Dry Ashing and Wet Oxidation Methods. The Case of the Rice Husk (*Oryza sativa* L.)

XIUPING SUN,[1,2] YAN WU,[1,2]* CHANGSUI WANG,[1,2] AND DAVID V. HILL[3]
[1]*The Laboratory of Human Evolution, Institute of Vertebrate Paleontology and Paleoanthropology, Chinese Academy of Sciences, Beijing 100044, China*
[2]*Department of Scientific History and Archaeometry, Graduate University of Chinese Academy of Sciences, Beijing 100049, China*
[3]*Department of Sociology, Anthropology, and Behavioral Science, Metropolitan State College of Denver, Denver, Colorado 80202*

KEY WORDS phytolith; rice husk; dry ashing; acid extraction

ABSTRACT Experiments were conducted to determine the effects that different methods for the preparation of modern plant samples have on the resulting phytoliths using rice husks (*Oryza* sp.). The methods that are commonly used in phytolith extraction include (1) dry ashing, (2) acid extraction (3) a combination of both techniques. The results showed that processing methods have an impact on the morphology of two subspecies of rice phytoliths, dry ashing producing more conjoined cell phytoliths or multicells phytoliths than acid extraction. Using a combination of both methods resulted in the presence of fewer conjoined cells than dry ashing alone, but more conjoined cells than acid extraction. Alternative explanations are proposed to explain the formation of conjoined phytolith cells. *Microsc. Res. Tech. 75:1272–1276, 2012.*

INTRODUCTION

The analysis of phytoliths is an important method for understanding the relationship between ancient people and plants, particularly those interested in the origin and development of agriculture (Fuller, 2009; Irarte et al., 2010; Mbida, et al., 2006; Denham et al., 2003; Piperno, 1988, 2006; Zhao et al., 1998). In the analysis of ancient phytoliths, modern plant reference material is indispensable, making it crucial to get the truly representative reference phytoliths. Currently, two basic methods are used to extract phytoliths from modern plants; dry ashing and acid extraction (Parr et al., 2001). It has been suggested that the two different methods probably produce different results in the ratios of isolated single cells and clusters of cells of phytoliths (Jenkins, 2009; Jones and Milne, 1963; Rovner, 1983). This study compares the two methods of phytolith extraction using different *Oryza sativa* subsp., and compares the results of each technique on the type and condition of the recovered phytoliths.

The effect of dry and wet ashing procedures for the extraction of phytoliths from modern plant samples has been discussed for several decades (Jenkins, 2009; Jones and Milne, 1963; Parr et al., 2001; Rovner, 1983). Jones and Milne (1963) compared the results of dry ashing and acid extraction on phytoliths from oats (*Avena sativa* L.). They found that the phytolith assemblages obtained using the dry ashing method contained carbon and a great deal of Na, K, Ca, and Mg. Both methods resulted in deferring the dehydration of the recovered phytoliths. The phytoliths from the dry ashing samples consisted of "large congeries" while the acid extraction produced single isolated phytoliths. Jones and Beavers (1964) observed that the dry ashing method could cause the fusion of phytoliths during heating, though they do not state what temperatures they employed in their method of phytolith preparation. Raeside (1970) found that dry ashing at 600°C produced a greater number of weakly silicified phytoliths than acid digestion. The presence of Na, K, Ca, and Mg would have acted as fluxing elements for the silica in the phytoliths thus lowering their melting point resulting in fusion of phytoliths into masses of cells. Without quantitative data regarding the amounts of these potential fluxing elements in relation to the silica and information of the firing temperature of the original study we cannot know how the fusion of phytoliths is affected by these elements.

Parr et al. (2001, 875-886) directly compared the effect of dry ashing and acid extraction on single celled phytoliths. Monitoring the effects of shrinkage and curvature found no significant differences between the two methods. They conclude that dry ashing "provides clean, lucid, disarticulated and in-situ phytolith assemblages more effectively than did the wet ashing method." Emma Jenkins (2009) showed that phytoliths from durum wheat [*T. turgidum* L. subsp. *durum* (Desf.) Husn.] using dry ashing produced more conjoined cells. On the basis of her analysis, she suggested that the generation of large multicelled phytoliths is related to elevated rates of silica precipitation which were believed to result from fast transpiration rates in arid and semiarid regions.

It is important to explore how different extraction methods affect the ratio of single cells to multicelled

*Correspondence to: Dr. Yan Wu, The Laboratory of Human Evolution, Institute of Vertebrate Paleontology and Paleoanthropology, Chinese Academy of Sciences, Beijing 100044, China. E-mail: yanyanwu3@gmail.com

Received 2 November 2011; accepted in revised form 20 March 2012

Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 41002057; Contract grant sponsor: the Knowledge Project of the Chinese Academy of Sciences; Contract grant number: KZCX2-YW-Q1-04; Contract grant sponsor: the CAS Strategic Priority Research Program Grant; Contract grant number: XDA05130501; Contract grant sponsor: the President Funding of the Graduate University of the Chinese Academy of Sciences

DOI 10.1002/jemt.22060

Published online 17 April 2012 in Wiley Online Library (wileyonlinelibrary.com).

TABLE 1. Methods used for the recovery of rice phytolithis

Dry ashing	Acid extraction
1. Weigh 0.3 g rice husk in the balance	1. Weigh 0.3 g rice husk in the balance
2. Put the sample in an empty crucible	2. Put the sample in a 50-ml beaker
3. Ash the sample in muffle furnace for 12 h at 550°C	3. Add 20 ml concentrated nitric acid and warm bath for more than 12 h
4. Transfer ashed samples into centrifuge tube	4. Transfer into a 30 ml centrifuge tube
5. Add HCl 10% (up to 6 ml) and shake tube	5. Add distilled water (up to 30 ml)
6. Wait for 5 min	6. Centrifuge 10 min at 2000 rpm
7. Level the sample with distilled water (up to 10 ml), tighten lid and shake tubes	7. Discard supernatant; repeat three times
8. Centrifuge 8 min at 3000 rpm	8. Air-dry the sample
9. Discard supernatant, repeat three times	9. Weigh the sample and record
10. Air-dry the sample	
11. Weigh the sample and record	

TABLE 2. Results of the different methods used to recover phytoliths

		Percent of forms in each conjoined category		
	No. of conjoined cells	Dry ashing	Dry ashing + acid extraction	Acid extraction
Oryza sativa L. subsp. *indica* Kato	Single cell	53.862	80.223	96.976
	2–5	39.024	16.503	2.827
	6–10	5.691	2.554	0.197
	11–20	1.423	0.720	0
	>20	0	0	0
Oryza sativa L. subsp. *japonica* Kato	Single cell	47.281	57.063	93.137
	2–5	48.387	37.220	5.331
	6–10	2.031	3.475	0.803
	11–20	2.031	1.682	0.730
	>20	0.276	0.560	0

phytoliths. Although great progress has been achieved in identifying potential formation mechanisms of multicelled phytoliths of cultigens that originated in West Asia (Gopher, 2001), the effects of different extraction techniques of rice (*Oryza Sativa* L.) phytoliths has not been studied. In this article, the effects of different methods for extracting phytoliths from two modern rice subspecies' husks will be compared. The possible formation mechanisms and the characteristics of conjoined phytoliths will also be discussed.

MATERIALS AND METHODS

Samples source: *Oryza sativa* L. subsp. *indica* Kato from modern cultivate fields in Zhejiang Province; and *Oryza sativa* L. subsp. *japonica* Kato from the Agricultural Research Institute of Anhui Province.

Laboratory method: For the O. *indica* specimen from Zhejiang Province: Initially 0.6 g of rice husks was prepared for phytolith analysis. The rice husks were washed three times in distilled water and dried in a cupboard at 30°C. The husks were then taken through the preparation processes outlined in Table 1. Our methodology followed that described by Piperno (2006:97-98). Another 0.3 g rice sample of husk was analyzed to examine the effects of oxidants on both methods. This study was done by using the processes outlined using steps 1-5 (Table 1) of the dry ashing and then 3-9 of the acid extraction. The O. *japonica* specimens were prepared using the same laboratory procedures O. *indica* sample described previously.

After the extraction, 0.15 mg of phytoliths from each extraction were mounted and recorded on a microscope slide. The slides were counted using a Nikon ECLIPSE LV100 POL microscope at 500X. Double peaked glume cells characterize the morphology of modern rice husk phytoliths. Phytoliths were counted according to the number of identifiable double peaked glume cells in each conjoined form using the broad counting categories: single cell, 2-5, 6-10, 11-20, >20.

RESULTS AND DISCUSSION

Results

The results of the application of the three methods used to produce rice phytoliths are presented in Table 2 and in Figure 1. For O. *indica* acid extraction produced 96.976% of single cells with double peaked glume, much more than was produced using the dry ashing method at 53.862%. Only 3.024 percent multi-celled phytoliths were observed using the acid extraction method. When the phytoliths from the rice husk sample were extracted using the combined dry ashing and acid extraction methods, the ratio of multicelled phytolith and single cells fell between the samples that were extracted using only a single technique. Using the combined method for phytolith production resulted in recovery of 80.223% conjoined phytoliths.

The application of the three phytolith preparation techniques using O. *japonica* produced similar results using the dry ashing and acid extraction techniques that were observed in the O. *indica* sample. Phytoliths extracted from the O. *japonica* sample using both the dry ashing and acid extraction methods also produced percentages of conjoined phytoliths that fell between either of the techniques when used alone. Although these two subspecies of rice produced slightly different results, this study reached following conclusions:

1. The dry ashing method of phytolith extraction produced more conjoined cells.
2. The acid extraction method produced more single cell phytoliths than conjoined cells.

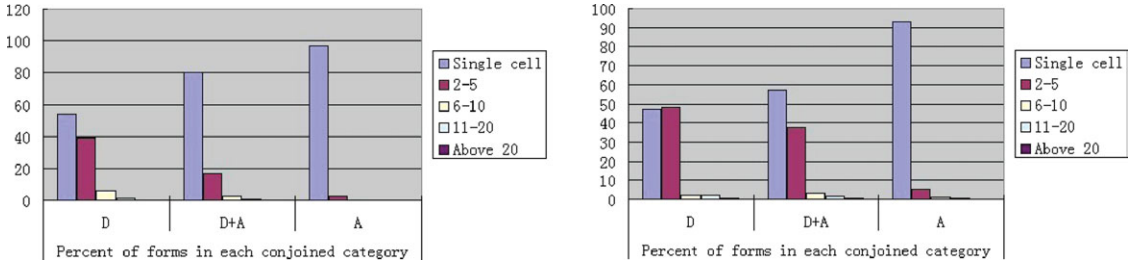

Fig. 1. Comparison of results of the three different phytolith extraction methods. The left is O. *indica* and the right is O. *japonica*. D is for dry ashing, A is for acid extraction. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

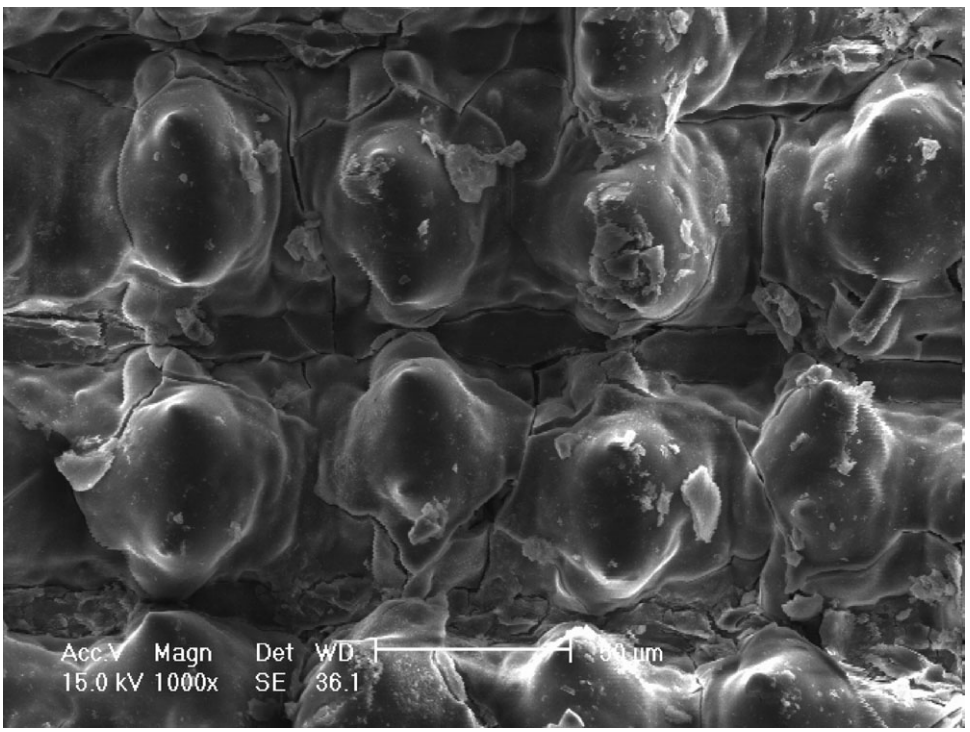

Fig. 2. The natural surface structure of rice lemma by scanning electronic microscope.

3. In the sample of phytoliths obtained using the dry ashing method and then using concentrated nitric acid, the ratio of the multicell phytoliths fell between the ratios of dry ashing and acid extraction techniques.

DISCUSSION

It is clear that different extraction methods result in different amounts of conjoined and single celled phytoliths from the two rice subspecies. As the result of this study several questions should be discussed: (1) What is the formation mechanism of conjoined cells (2) What materials combined the cells and (3) Can cells be separated by chemical reagents or other methods.

In our experiments, the two kinds of rice used in this experiment were grown under similar conditions using irrigation-based agriculture. Figure 2 shows the surface of rice lemma by scanning electronic microscope with the double peaked glume cells arranged regularly in horizontal and vertical rows. The image of rice husk phytoliths (Fig. 3) shows that the base of double peaked glume cells is the locations where the cells are joined. In the sample of phytoliths extracted using a combination of the two methods, some multicelled phytoliths breakdown and the ratio of single cells increased suggesting that the use of a strong oxidant may have separated the conjoined cells. Based on the current study the amount of conjoined cells is more likely the result of differences in the methods used to prepare the phytolith samples than to the growing conditions of the rice. One explanation for the presence of increased single cells is that during acid extraction the oxidation of the organic matter weakens and dissolves the material that joined the cells. The centrifuging process forces the phytoliths apart resulting in the mechanical breakdown of conjoined forms. Alternatively, as proposed by Jones and Milne (1963), dry ashing may cause the

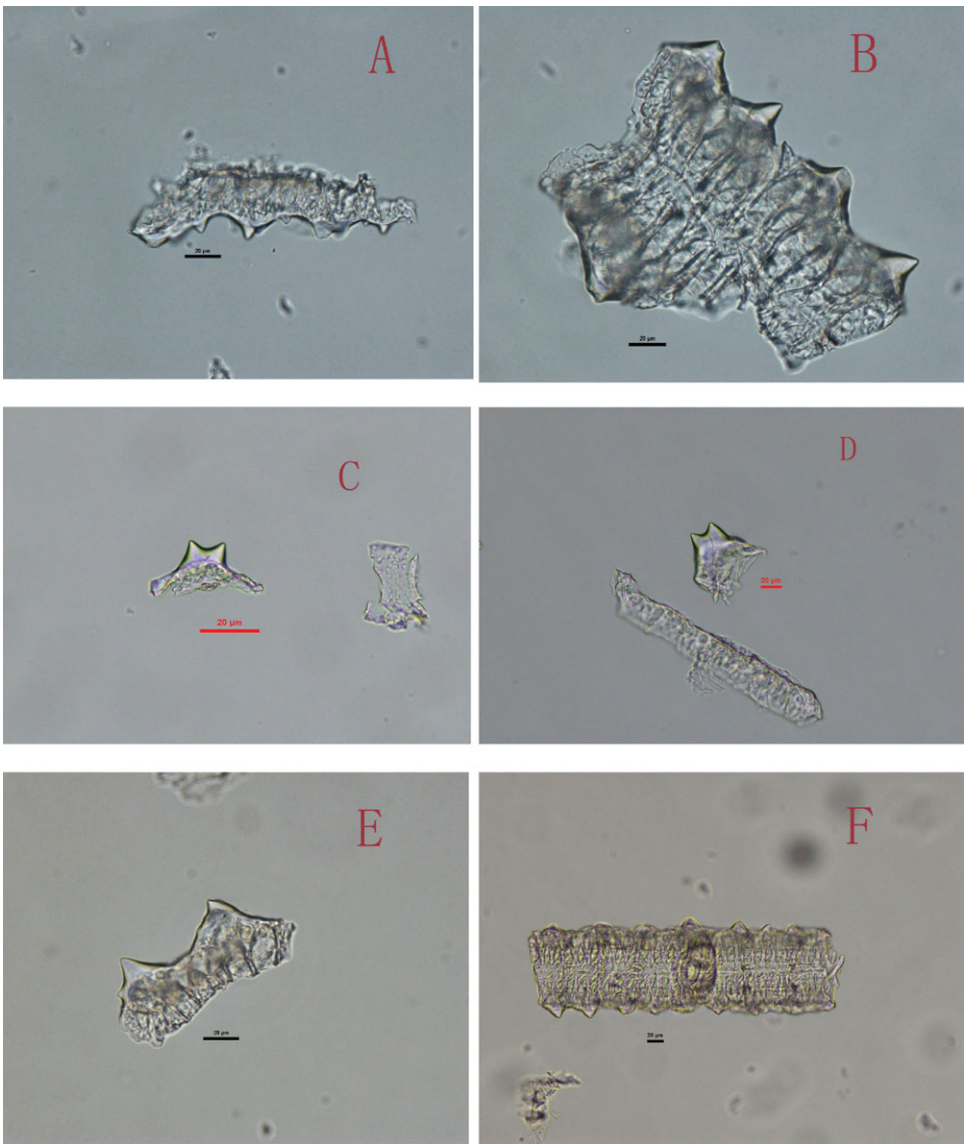

Fig. 3. Images of *Oryza sativa* L. subsp. *indica* Kato phytoliths produced using the three processing methods: (**A**) and (**B**) are from dry ashing, (**C**) and (**D**) are from acid extraction, (**E**) and (**F**) are produced by both methods. (C) and (D) are single cells, (A) and (E) are 2-5 type, (B) is 6-10 type, (F) is ranked in 10-20 type. (F) is viewed at 20×, others are viewed at 50×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

silica to dehydrate resulting in the fusion of individual phytolith cells. It is also possible that the presence of fluxing agents such as Na, K, Ca, and Mg lower the melting point of the silica in the phytolith cells. The temperature reached during the heating step of the dry ashing process is sufficient to fuse the cells together. The material that combines single cells should be examined through future research.

As we can find double peaked glume cells in archaeological samples, and we know that high temperature may result in the formation of conjoined cells. Rice phytoliths recovered from archaeological sites may have become conjoined through cultural or natural processes. Further research regarding how phytoliths may have been joined in antiquity from those joined during sample preparation is required. Further experimentation is also required to explore the mechanisms that govern the formation of multicelled phytoliths.

CONCLUSION

A comparison was made of two standard laboratory techniques for phytolith extraction using modern *Oryza sativa* L. subsp. *indica* Kato and modern *Oryza sativa* L. subsp. *japonica* Kato. Dry ashing produced more conjoined phytolith cells while acid extraction results in a larger percentage of single cells. The percentage of single to conjoined phytoliths that were produced using a combination of the two techniques fell between either of the techniques when used alone. These results demonstrate that both dry ashing and acid extraction techniques affect the presence and

number of conjoined forms of *Oryza sativa* L. two subspecies.

REFERENCES

Denham TP, Haberle SG, Lentfer, C, Fullagar R, Field J, Therin M, Porch N, Winsborough B. 2003. Origins of agriculture at Kuk Swamp in the Highlands of New Guinea. Science 301:189–193.

Fuller DQ, Qin L, Zheng Y, Zhao Z, Chen X, Hosoya LA, Sun G-P. 2009. The domestication process and domestication rate in rice: Spikelet bases from the Lower Yangtze. Science 323:1607–1610.

Gopher A, Abbo S, Lev-Yadum S. 2001. The "when", the "where" and the "why" of the Neolithic Revolution of Levant. Documenta Praehistorica XXVIII:UDK 903′12/′15(5–11)634.

Iriarte J, Glaser B, Watling J, Wainwright A, Jonathan J, Renard D, Rostain S, Mckey D, 2010. Late Holocene Neotropical agricultural landscapes: phytolith and stable carbon isotope analysis of raised fields from French Guianan coastal savannahs. J Archaeol Sci 37:2984–2994.

Emma J. 2009. Phytolith taphonomy: a comparison of dry ashing and acid extraction on the breakdown of conjoined phytoliths formed in *Triticum durum*, J Archaeol Sci 36:2402–2407.

Jones LHP, Milne AA. 1963. Studies of silica in the oat plant: 1. Chemical and physical properties of the silica. Plant Soil 18:207–220.

Jones RL, Beavers AH. 1964. Aspects of caternary and depth distribution of opal phytoliths in Illinois soils. Soil Sci Soc Am Proc 28:413–416.

Mbida CH, De Langhe E, Vrydaghs L, Doutrelepont H, Swennen Ro, Van Neer W, de Maret P. 2006. Phytolith evidence for the early presence of domesticated banana (Musa) in Africa. In: Zeder MA, Bradley DG, Emshwiller E, Smith BD, editors. Documenting domestication. New genetic and archaeological paradigms. Berkeley, Los Angeles, London: University of California Press. pp. 68–81.

Parr JF, Lentfer CJ, Boyd WE. 2001. A comparative analysis of wet and dry ashing techniques for the extraction of phytoliths from modern plant material. J Archaeol Sci 28:875–886.

Piperno DR. 1988. Phytolith Analysis: An Archaeological and Geological Perspective. London: Academic Press.

Piperno DR. 2006. Phytoliths: A comprehensive Guide for Archaeologists and Paleoecologists. Oxford: Altamira Press.

Piperno DR, Pearsall DM. 1998. The Silica Bodies of Tropical American Grasses: Morphology, Taxonomy, and Implications for Grass Systematics and Fossil Phytolith Identification. Washington, D.C.: Smithsonian Institution Press.

Raeside JD. 1970. Some New Zealand plant opals. NZ J Sci 13:122–132.

Rosen AM. 1992. A Preliminary identification of silica skeletons from Near Eastern archaeological sites: an anatomical approach. In: Rapp G Jr, Mulholland SC, editors. Phytolith Systematics, Emerging Issues. Advances in Archaeological and Museum Science No.1. New York: Plenum Press. pp. 129–147.

Rosen AM, Weiner S. 1994. Identifying ancient irrigation: A new method using opaline phytoliths from emmer wheat. J Archaeol Sci 21:125–132.

Rovner I. 1983. Plant opal phytolith analysis: major advances in archaeobotanical research. Adv Archaeol Meth Theory 6:225–266.

Webb EA, Longstaff FJ. 2002. Climatic influences on the oxygen isotopic composition of biogenic silica in prairie grass. Geochim Cosmochim Acta 66:1891–1904.

Zhao Z, Pearsall DM, Benfer RA Jr, Piperino DR. 1998. Distinguishing rice (*Oryza stiva* Poaceae) from wild *Oryza* species through phytolith analysis: finalized method. Econ Bot 52:134–145.