Convenient Estimation of Unfertilized Grains in Rice

Tohru Kobata, Yoshio Akiyama and Takuya Kawaoka

(Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu-cho, Matsue, Shimane 690-8504, Japan)

Abstract: Failure of fertilization in rice is a critical yield-determining factor in plants subjected to temperature or water stress at the early-reproductive stage and in high-yield cultivars bearing heavy spikelets. Although it is important to identify quickly the unfertilized spikelets for research and selection of stress-resistant or high-ripening cultivars from bulk samples, the identification takes time because unfertilized spikelets are usually determined by visual and manual procedures. Our objective was to develop a convenient method to identify unfertilized spikelets in rice. Takanari spikelets at maturity grown in the paddy field were separated into floating and sinking spikelets by different specific gravity solutions of ethanol/water mixture. The unfertilized spikelets were identified by checking the grains inside the spikelets by light penetration and examining the spikelets manually. The percentage of floating spikelets decreased with the increase in ethanol concentration, and that of floating spikelets approximately coincided with the percentage of unfertilized spikelets when the specific gravity was below $0.90 \times 10^3 \text{ kg m}^{-3}$, corresponding to over 70% -ethanol. In a practical range of temperature the specific gravity scarcely changed. In an 80% -ethanol solution, the percentages of floating spikelets in Takanari grown under different nitrogen applications and in rice cultivars having different spikelet size approximately coincided with percentages of unfertilized spikelets, though the percentages of floating spikelets was 5 to 7% higher than the unfertilized spikelets. The use of 70% -ethanol solution increased the difference in some rice cultivars. We concluded that the gravitation method would be convenient for identification of unfertilized spikelets in bulk samples of rice.

Key words: Cultivar, Gravitational method, Rice, Ripened grain, Sterility, Stress, Unfertilized spikelet.
(2) Core Collection cultivars
From 69 rice cultivars of the NIAS Global Rice Core Collection that covers 90% of diversity in allelic gene in rice (Kojima et al., 2005; NIAS 2009), 49 cultivars that can head and mature under natural day length condition in Matsue were sown in the rice seedling tray with rice seedling soils and transplanted to the paddy field at Shimane University Experimental Farm (9 m above sea level, 133°E, 35°N) on 13 May 2008. Seedlings were planted in rows 0.30 m apart at a spacing of 0.15 m and each cultivar occupied 0.75 m a row. Fertilizer N [as (NH4)2SO4], P (as CaHPO4), and K (as KCl) was applied at rates of 0.2 g N m⁻², 0.2 g P m⁻², and 0.2 g K m⁻², respectively, as basal dressing and at a rate of 0.5 g N m⁻² as top dressing at the interval of two weeks until the last cultivar headed. Soil conditions and fertility in the experimental field were carefully equalized and managed.

(3) High temperature treatments
Four rice cultivars, including Japanese japonica cultivar (Koshikihikari), indica cultivar (IR72), and two improved indica japonica cultivars (Takanari) in Japan and (Miyang23) in Korea were grown in 4 liter pots containing black soil (Andosol) and sandy soil (Masatsuchi) mixture at Shimane University Experimental Farm in 2008. Twenty germinated seeds were sown in the pots in a circle on 12 April 2008 (IR72), 7 May 2008 (Takanari and Miyang23), and 19 May 2008 (Koshikihikari). Fertilizer N [as (NH4)2SO4], P (as CaHPO4), and K (as KCl) were applied at rates of 0.2 g N m⁻³, 0.4 g P m⁻³, and 0.6 g K m⁻³, respectively, as basal dressing and at rates of 0.2 g N m⁻³ as top dressing before heading. To obtain uniform panicles, we removed tillers that appeared after sowing and only kept the main culm (Satake, 1972).

At heading, pots of Koshikihikari were transferred into the temperature-gradient chamber on 12 August, and the pots of the other cultivars on 18 August 2008. Steel frames 2.1 m high, 2.2 m wide, and 14 m long covered with a thermoplastic fluoropolymer film of (a copolymer comprised of tetrafluoroethylene and ethylene, FTFE, Asahi Glass Green-Tech, Tokyo) and was placed in the field at Shimane University. One end of the tunnel frame was furnished with a 20-cm-diameter fan, and two 25-cm-diameter fans for ventilation, and the other end was left open. The 20 cm fan was operated all day, but the 25 cm fans were controlled with a thermo controller (Noden-thermo, ND-610, Tsukuba Electric Co., Tokyo). The controller switched the ventilation fans on when temperature above the plants exceeded set temperatures. Air from the open side of the tunnel was warmed by solar radiation, and the temperature in the tunnel gradually increased.
increased from the open end to the closed end of the chamber. Carbon dioxide concentrations in the chamber monitored with an infrared gas analyzer had decreased by less than 2%, compared to ambient air at noon on fine days. Photosynthetic active radiation inside the tunnel was 14% less than in ambient. Three pots of each cultivar were placed at distances of 14, 7, and 1 m from the open air inlet as high, middle, and ambient temperature plots, respectively. The temperature in each location was monitored every 30 min with a data logger and ventilated thermometer. The temperature treatments were replicated three times by three chambers.

2. Identification of unfertilized spikelets by the gravitational method

(1) Test of selection by different specific gravity solutions

Above-ground plants of ten adjustment hills in Takanari at the field site were harvested at maturity and air dried in a glasshouse for a week. The spikelets were removed from plants and selected by the gravitational method using a 1.06 x 10^3 kg m^-3 salt solution of (NH₄)₂SO₄ to select ripened spikelets (Matsushima and Yamaguchi, 1952; Yoshida et al., 1976). Spikelets were divided into ripened and floating spikelets including fertile and unfertilized spikelets. The ripened and floating spikelets were counted with a grain counting machine (RC-2501, Nippon Plant Seeder Co., Ltd, Tokyo). Samples of a conventional nitrogen application plot [a-4:2:6 g m^-2] were used. From the floating spikelets, 500 spikelets were randomly taken, were soaked in the ethanol solution with different specific gravities to divide them into floating and sinking spikelets. Synthetic ethanol (>99.5%, Japan Alcohol Sale Corporation, Tokyo) was mixed with water at a rate of 100/0, 80/20, 60/40, 40/60, and 0/100, whose specific gravity was 0.796, 0.856, 0.907, 0.948, and 0.999 x 10^3 kg m^-3 (room temperature of 20.3ºC), respectively. Specific gravity was measured with a gravity meter (DA-130N, Kyoto Electronics, Kyoto). The specific gravity of the five series of ethanol solution was measured at different temperatures in the temperature controlled incubator. Within one minute, spikelets were clearly divided into floating and sinking spikelets. These spikelets were air dried and counted by hand to determine the percentage of floating spikelets (%FLS).

Fig. 1. Percentage of floating spikelets (%FLS) in Takanari rice cultivars and the specific gravities of ethanol solution. The specific gravity of 1.06 x 10^3 kg m^-3 was obtained using ammonium sulfate solution. Each data point is the average ±standard deviation of three replicates. Samples were grown rice under paddy conditions of [a 4-2-6 g m^-2] (Table 1). The lateral bar indicates the mean of %UFS, and the vertical bar is the range of 95% reliability of the mean.

(2) Selection of unfertilized spikelets

Fertilized and unfertilized spikelets for all samples were identified by checking the grains inside the spikelets on the illuminated light box (Color Illuminator, Fuji Color Co., Tokyo) and by crushing the grains with fingers. The empty spikelets/total spikelets were regarded as the percentage of unfertilized spikelets (%UFS). After the selection with ethanol solution, unfertilized spikelets included in the sinking spikelets and fertilized spikelets included in the floating spikelets were counted separately.

Fig. 2. Relationship between specific gravity (SG) and volume ratio of ethanol (99.5%) to water (V/V₀) in different temperature solutions. The mesh indicates a range that %FLS approximately coincided with %UFS in Figure 1. When SG=aV/V₀+bV/V₀+c, a=-4.46 x 10^-3, b=-5.67 x 10^-3, and c=-0.99 (R²=0.999) under 16.5ºC, a=-4.44 x 10^-3, b=-4.37 x 10^-3, and c=-0.99 (R²=0.999) under 20.4ºC, a=-1.45 x 10^-3, b=-6.49 x 10^-3, and c=-1.00 (R²=1.000) under 24.4ºC, and a=-1.43 x 10^-3, b=-7.07 x 10^-3, and c=1.00 (R²=1.000) under 28.6ºC.
3. Comparison between percentages of unfertilized and floating spikelets

Samples in all treatment plots in the field were divided into sinking and floating spikelets in 80% ethanol solution. In the pot experiment in the high temperature treatment, three plants from each pot in the same temperature plot were sampled, and soaked in 70% ethanol solution to separate into floating and sinking spikelets.

**Results**

1. Optimum specific gravity for identification of unfertilized spikelets

When Takanari spikelets were soaked in five different specific gravity solutions of ethanol/water mixture, the percentage of floating spikelets (%FLS) decreased as the ethanol concentration increased (Fig. 1). The average percentage of unfertilized spikelets (%UFS) in this sample was 23%, and the 95% confidence range was 21.5–24.1%.

Fig. 1 showed that %FLS in the ethanol solution with a specific gravity of around $0.9 \times 10^3$ kg m$^{-3}$ approximately coincided with %UFS. The specific gravity of ethanol solution decreased with an increase in ethanol concentration, and the relationship between specific gravity and ethanol concentration was nearly the same between 17ºC and 29ºC (Fig. 2). Therefore, the concentration of the ethanol solution with the above-mentioned specific gravity of $0.9 \times 10^3$ kg m$^{-3}$ was about 70%.

The sinking spikelets included some unfertilized spikelets, which increased slightly with a decrease in the specific gravity of the solution (Fig. 3). The floating spikelets also included some fertilized spikelets, in which small and thin grains were observed, and the percentage of fertilized spikelets (%FS = 100-%UFS) decreased with a decrease in specific gravity (Fig. 3). In the solution with a specific gravity below $0.9 \times 10^3$ kg m$^{-3}$, %FLS was 23%, which approximately coincided with %UFS. At this specific gravity, %UFS in the sinking spikelets was 2% and %FS in the floating spikelets was 3%. Hence the error of the identification of unfertilized spikelets by this method was ±3%.

**Fig. 3.** Percentages of sinking and unfertilized spikelets (%SS and %UFS) (upper) and floating and fertilized spikelets (%FLS and %FS) (lower) in spikelets of Takanari soaked in different concentrations of ethanol. Each data point is the average ± standard deviation of three replicates in the samples for Figure 1.

**Fig. 4.** Relationship between the percentage of unfertilized and floating spikelets (%UFS and %FLS) in Takanari grown under eight different nitrogen application conditions. The floating spikelets were separated by soaking in 80%-ethanol solution. Alphabets and numbers of the symbols indicate the total amounts and dressing times of nitrogen fertilizer shown in Table 1. The dotted line shows equivalence between the two percentages.

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 Yield components including percentage of ripened grain (%RG), ripened and total grain yield (dry matter), percentage of fertilized spikelets (%FS), percentage of sinking spikelets (%SS) in 80%-ethanol solution, a contribution of unfertilized spikelets (%UFS) to un-ripened spikelets (CUS = [(100-%FS) / (100-%RG)]) and floating spikelets (%FLS) to unfertilized spikelets (CUS = [(100-%SS) / (100-%RG)]) under eight different nitrogen fertilizer conditions in Takanari rice cultivar. Different amounts of nitrogen fertilizer were applied at different times (a: transplanting-tillering-flower initiation or b: transplanting-tillering-forty days before heading, g m$^{-2}$). Number in parentheses is total amount of nitrogen.

| Nitrogen treatment | Spikelet number | 1000 grain weight | Ripened grain yield | Total grain yield | Percentage of fertilized spikelets** (%FS) | Percentage of %SS | CUS | %FS vs %SS |
|--------------------|-----------------|------------------|--------------------|------------------|------------------------------------------|------------------|-----|-----------|
| a 4-2-2 (8)        | 70036†          | 63.8             | 15.5               | 695              | 771                                       | 76.6             | 75.6 | **§0.65   |
| b 0-0-8 (8)        | 59705†          | 67.1             | 15.6               | 626              | 668                                       | 77.2             | 76.1 | 0.69 0.73 |
| Average            | 66995‡          | 62.8             | 15.4               | 651              | 718                                       | 76.3             | 74.6 | 0.64 0.68 |
| SE                 | 4068            | 2.7              | 0.3                | 48               | 51                                        | 0.0              | 0.0  |

* Percentage of ripened grain (%RG) was determined in the solution with a specific gravity of 1.06 ×10$^{-3}$. 
** Percentage of fertilized spikelets (%FS) is 100-%UFS. 
† Percentage of sinking spikelets (%SS) is 100-%FLS. 
§ † indicates significant difference between %FS and %SS at 0.05, ** 0.01 and ns not significant.

2. Estimation of the percentage of unfertilized spikelets by the gravitational method

(1) In the same cultivar under different nitrogen applied conditions

In cv. Takanari, in eight nitrogen application treatments, the relationship between %UFS and %FLS in 80% ethanol solution was shown (Fig. 4). The %FLS was similar to %UFS, although the former was about 7% higher than the latter (Fig. 4). In this experiment, with different amounts of nitrogen and methods of nitrogen application, the spikelet number, percentage of ripened grain (%RG), ripened grain yield (commerce yield at dry matter), and total grain yield (bulk yield) was 60000–73000 m$^{-2}$, 57.8–67.1%, 549–695 g m$^{-2}$, and 604–771 g m$^{-2}$, respectively (Table 1). The percentage of sinking spikelets (%SS=100-%FLS) by the gravitational method in each treatment plot was 3–4% lower than %FS and the average %SS was significantly lower than %FS in most of the nitrogen treatment plots (Table 1). Unfertilized spikelets (%UFS) and %FLS occupied 64 and 68% of the unripened grains (100-%RG), respectively. Hence, the lower percentage of ripened grain in Takanari than in other cultivars (Kobata and Uemuki, 2006) resulted dominantly from unfertilized spikelets.

(2) In different cultivars with diverse variations in spikelet sizes and weights

The solution necessary to obtain unfertilized floating spikelets was 80% ethanol in Takanari but the optimal ethanol concentration for the other cultivars with different spikelet sizes and weights should be tested. From 49 cultivars in the Core Collection, 10 cultivars covering the diverse variance in the husk size were selected (NIAS, 2009) and the spikelet number, 1000 spikelet dry weight, and %UFS and %FLS were measured (Table 2). The heading date was between 16 July and 13 August. The 1000 spikelet dry weight of the ten cultivars varied between 16.8 and 29.7 g and spikelet number per panicle between 64 and 176. Although 1000 spikelet weight was lightest in No 15 and heaviest in No 31, the percentage of the husk to the fertilized spikelet dry weight was similar (18 and 19%). The %UFS was between 3.8 and 29.4 %, and the %FLS in 80% ethanol solution was between 4.4 and 31.0 %. Thus, %UFS and %FLS showed a large variance among 10 cultivars (CV=0.62) and no significant difference between %FLS and %FS was observed in any of the cultivars except for cultivar No 7.

There was a close relationship between %UFS and %FLS in the 10 cultivars, although %FLS was 4.4 % higher than %UFS (Fig. 5). Thus, the relationship was similar regardless of spikelet size or dry weight of the cultivars.
Furthermore, awn length longer than 4 cm such as cultivar No. 2 did not affect the relationship.

(3) In cultivars subjected to different high temperatures at anthesis

Four rice cultivars were placed at three different positions in the temperature gradient chamber, near the outside, in the middle, and inner part, where the average temperature during 36 days after heading was 25.2, 26.1 and 26.6°C, respectively, and the average daily maximum temperature was 30.9, 33.0 and 34.4°C, respectively. Koshihikari was subjected to a slightly higher temperature than the other cultivars because it headed one week earlier than the others. The %UFS in most cultivars decreased by 12–30% with an increase in temperature although the degree of decrease was smaller in some cultivars.

Spikelets at maturity were soaked in 70% instead of 80%-ethanol solution to save the cost, and %FLS and %UFS were compared. There was a close correlation between them in the four cultivars (Fig. 6), although the coefficient of the correlation was lower in Milyang23 than in the other cultivars because of the narrow variation in observed data. The coefficient of the equation between %UFS and %FLS in the four cultivars was 0.819 to 0.992, while it was lower in Takanari and Milyang23 (Fig. 6). The average of the coefficients for all cultivars was 0.932 and %FLS was 7% higher than %UFS on average in the

Table 2. Spikelet size, number of spikelets, 1000 spikelet dry weights and percentage of unfertilized and floating spikelets (%UFS and %FLS) in 80% ethanol solution in ten rice cultivars. These rice cultivars were selected from 49 cultivars of Core Collection (RFLP-based Rice Diversity Research Set of Germplasm) (NIAS, 2009) that headed and matured under natural light conditions in Matsue showing diverse differences in size and weight of spikelets.

| Cultivar No | Origin | Length of spikelet* (mm) | Width of spikelet (mm) | No. of spikelets per panicle** | 1000 spikelet weights (g) | Unfertilized spikelets (%UFS) | Floating spikelets (%FLS) | Unfertilized vs Floating spikelets |
|-------------|--------|--------------------------|------------------------|-----------------------------|--------------------------|-----------------------------|---------------------------|-------------------------------|
| 31 Bangladesh | 7.0 | 3.2 | 70.8 | 29.7 | 8.6 | 8.2 | ns† |
| 11 China | 7.0 | 3.5 | 176.4 | 25.7 | 7.0 | 7.3 | ns |
| 45 Myanmar | 5.5 | 2.9 | 126.6 | 23.4 | 11.3 | 11.3 | ns |
| 7 Philippines | 5.9 | 3.3 | 117.8 | 23.4 | 12.8 | 13.9 | * |
| 10 China | 6.7 | 2.9 | 131.4 | 22.1 | 16.0 | 16.4 | ns |
| 37 India | 5.1 | 3.1 | 135.4 | 19.8 | 6.5 | 7.5 | ns |
| 38 India | 6.3 | 2.8 | 64.4 | 18.9 | 18.4 | 19.3 | ns |
| 2 India | 6.9 | 2.6 | 132.4 | 18.6 | 3.8 | 4.4 | ns |
| 22 Philippines | 5.4 | 3.1 | 130.0 | 17.9 | 29.4 | 31.0 | ns |
| 15 India | 6.1 | 3.1 | 132.0 | 16.8 | 8.4 | 8.6 | ns |
| Average | 6.2 | 3.1 | 121.7 | 21.6 | 12.2 | 12.8 | |

* Length and width of spikelet are from data in RFLP-based Rice Diversity Research Set of Germplasm (NIAS, 2009).
** No of spikelets and 1000 grain weight was mean of five panicles.
† * indicates significant difference at 0.05 level between %UFS and %FLS and ns not significant.
‡ cv is coefficient of variation.

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![Graph showing relationship between percentage of unfertilized and floating spikelets (%UFS and %FLS) in ten rice cultivars selected from the Rice Core Collection. The floating spikelets were separated by soaking in 80%-ethanol solution. Number of each symbol indicates cultivar. For origin of the cultivar and the spikelet size see Table 2. Open symbols indicate larger spikelet size cultivar (29.7 to 23.4 g of 1000-grain dry weight) and closed symbols smaller (22.1 to 16.8). The dotted line shows the equivalence between the two percentages.](image-url)
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70%-ethanol solution.
The sterility of spikelets in rice was affected by temperature in the morning, because flowering in most of the cultivars ends around noon (Satake and Yoshida, 1978; Nishiyama, 1983). Relationships between %SS or %FS and average temperature from 9 to 12 am (flowering time) for one week after heading are shown in Fig. 7. Both values decreased with the rise in temperature, although in Miliyan23 and Takanari the degree of decrease in %SS was slightly larger than that in %FS (Fig. 7).

Discussion
1. Optimum concentration of the solution for the selection of unfertilized spikelets
Most of the unfertilized spikelets were separated from the fertilized spikelets by soaking in an ethanol solution mixture (80%-ethanol solution) with a specific gravity of below 0.9 × 10^{-3} kg m^{-3}. The %FLS and %UFS were nearly the same (Fig. 1). However, the floating spikelets contained some fertilized spikelets and sinking spikelets some unfertilized spikelets. This caused an error in the estimation of %UFS from %FLS, but the error was less 3% (Fig. 3). In Takanari grown under several different nitrogen application conditions (Fig. 4) and in ten rice cultivars having different spikelet sizes and weights (Fig. 5), the %FLS was 5 to 7% higher than %UFS. The overestimation should be considered to be small for the field samples that variances around 5% or more are sometimes observed.

However, when lower 70%-ethanol solution was used for the selection of the floating spikelets in plants subjected to high temperatures, the overestimation in indica × japonica two cultivars (Takanari and Milyang 23) seemed to be higher than japonica and indicia cultivars (Fig. 6). The overestimation in %FLS tends to reduce with an increase in ethanol concentrations (Figs. 2, 3). This suggests that a higher concentration of ethanol solution such as 80% should be used for accurate separation of unfertilized spikelets.

By this method, the %UFS can be estimated by simply counting the floating spikelets, without time-consuming visual or manual observation. Furthermore, if an electric

Fig. 6. Percentage of unfertilized and floating spikelets (%UFS and %FLS) in four rice cultivars subjected to three different temperatures after heading with temperature gradient chambers. The floating spikelets were separated by soaking in 70%-ethanol solution. Each data is mean of three replications. The dotted line shows the equivalence between the two percentages.

Fig. 7. Percentage of sinking (upper) or fertilized (lower) spikelets (%SS and %FS) in four rice cultivars and average temperatures from 9 to 12 JST (Japan standard time) for one week after heading (flowering time). Data of %SS and %FS are from Figure 6. Each data point is the average ± standard error of three replicates.
grain-counting machine is available, the number of unfertilized spikelets even in bulk and diverse cultivar samples can be counted effectively.

2. Application of the method to laboratory work
The concentration of 80% ethanol is similar to that of a commercialized disinfectant ethanol (around 80%), and hence the solution can be easily used in the laboratory. Ethanol solution can be used without harmful effects, and can be easily disposed after the measurement.

3. Additional yield components of rice
The identification of unfertilized spikelets adds a valuable new yield component, percentage of sterility, to the ordinarily yield components (Yoshida et al., 1976; Yoshida, 1981). After spikelets at maturity are separated in the salt solution of 1.06 × 10^3 kg m^-3 to determine percentage of ripened grain (%RG) and 1000 ripened grain weight, the floating spikelets are divided into fertilized and unfertilized spikelets by the 80% ethanol solution. By this procedure harmful effects of ethanol solution on ripened seeds can be escaped, even if the seeds will be used for germination. The 100-%RG (percentage of waste grain) can be divided into two components, the percentages of fertilized but inadequate ripened spikelets and that of unfertilized spikelets (Table 1). These components would help determine which of sterility or assimilate supply limits the yield of rice subjected to water or temperature stress or in high-yielding rice cultivars.

4. Conclusion
We concluded that the gravitational method using an ethanol solution can be used to quickly and conveniently identify unfertilized spikelets in bulk samples such as field-grown plant materials or diverse rice cultivar sources. Ethanol solution of over 80% would be useful for separation of unfertilized spikelets with high accuracy.

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