Annals of Sri Lanka Department of Agriculture 2013. 15: 317-323

Short Communication

ETHANOL ASSAY AS A RAPID AND RELIABLE VIGOUR TEST FOR SEED PADDY (*ORYZA SATIVA* L.)

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INTRODUCTION

Good quality seed paddy (*Oryza sativa* L.) is an important basic requirement to obtain a healthy crop in paddy fields. Estimated seed paddy requirement in Sri Lanka for establishment in the year 2010 was about 0.1 t and, 15-20 % of the requirement was fulfilled (AgStat, 2012). Therefore, farmers produce their own seed paddy in their fields, out of which only 35 % of them found to be up to the quality standards. Seed paddy is stored for nearly 4 to 6 months until establishment of the next season. During seed storage, rapid seed deterioration has become a problem which is associated with seed vigour especially in the tropical climate with high temperature and high humidity (Siddique *et al.*, 1998). The ISTA germination test (1995) recommends a germination assay for 14 days but it is considerably, lengthy and also offers the germination percentage of the seed lot, but it will never forecast the actual seedling vigour. In contrast, seed vigour test is a more sensitive index for seed quality than germination test thus Delouche and Baskin (1973) proposed to develop the most sensitive vigour test based on membrane degradation that is the first sign of seed deterioration. Mitochondrial membranes are also deteriorated by oxidation during seed ageing (Benamar *et al.*, 2003). Seeds which have deteriorated mitochondria are able to produce metabolic energy from pyruvate by anaerobic fermentation at least on repair mechanisms during germination and the end product is acetaldehyde and ethanol in plants (Smith and Rees, 1979; Kennedy *et al.*, 1992). A correlation of ethanol production and decline of germination or seedling growth was observed. Measuring the developed ethanol could be used as an indicator to denote the germination as an alternative to the current germination assay. The feasibility of adopting such an ethanol test is understood by the introduction of breath analyzer technology as a rapid and inexpensive method for measurement of ethanol production in imbibed seeds. Therefore, there is a possibility to test vigour of paddy seeds during storage. Thus, the aim of the experiment was to develop rapid and quantitative assay for paddy seed vigour based on ethanol production of seeds.

MATERIALS AND METHODS

Preparation of seed samples and seed treatments

Seeds of variety At 362 (3 ½ month age class) were used for this experiment and the initial age of seeds was 30 days. Aged paddy seed samples were prepared using

different artificial ageing conditions such as elevated moisture level, humidity and temperature (using accelerated ageing [AA] condition).

In first seed treatment, 50 g of paddy seeds were placed in a small desiccator containing saturated NaCl to equilibrate seeds to 75% of RH level. The equilibrated relative humidity (eRH) level was recorded using a data logger which was placed in the desiccator. After 7 days the desiccator with seed samples was kept in an oven at 43 ^{0}C for 3, 5 and 7 days, respectively. Simultaneously 50 g of paddy seeds were placed in a desiccator with water until reach of RH to 100% for second seed treatment. Desiccator was lined with wet filter papers to attain required RH within a short period. Desiccator containing these seed samples was incubated in the oven at 100% RH and 43 ^{0}C of temperature for 2, 4 and 6 days (Gholami and Golpayegani; 2011). After incubation all seed samples were re-dried to 35% of RH and remained until the ethanol assay is commenced.

Ethanol Assay

Half gram (0.5 g) of each prepared seed samples were poured to 20 ml glass vials. Different volumes of water were added directly on seeds until it reach a moisture condition of 20 % and 30 % of final seed moisture content. This water requirement was calculated based on the initial moisture content (8 %) and oil content (17.5 %) of paddy seeds. After adding water, all vials were closed by aluminium crimp cap with rubber septum. Vials with each treatment were placed in ovens at 40 ^{0}C and 50 ^{0}C of temperatures. These treatments were replicated thrice and arranged in a complete randomized design (CRD). Then vials were incubated for 6 hours during the assay. At each hour, accumulated ethanol in head space of glass vials was recorded with the modified breath analyzer.

Germination test

Germination test was conducted to record the aged paddy seed germination which was influenced for aging under different aging conditions. One hundred seeds obtained from each ageing treatment and control (seeds equilibrated at 35 % of RH level) was placed on two standard blue germination papers moistened with 45 ml water. Small plastic trays (20 cm x 15 cm x 2.5 cm) with seeds were arranged in an incubator according to the complete randomized design with two replicates. The whole stacks of trays were covered by a polythene bag to maintain high humidity in the incubator. These seeds were incubated at 20 ^{0}C of temperature in 12 hours dark and 12 hours lightened conditions for 7 days (Hay *et al.*, 2012). Seed with a radical length of 4 mm was considered as a germinated seed and the count was recorded. Total germination and speed of the germination (t_{50}) were determined using GERMINATOR software developed by Joosen *et al.* (2010).

Determination of oxygen concentration

Parallel to the ethanol assay, measurements of oxygen concentration was also done to confirm the constant amount of oxygen availability in glass vials. Before sealing vials an oxygen sensor spot was placed on the top inside wall of glass vials with treated (aged and control) seeds. Oxygen concentration in sealed vials was measured using oxygen spots and fibre optic trace oxygen meter (Pre Sens Company, Germany) at room temperature. After 6 hours of incubation at 40 ^{0}C and 50 ^{0}C of temperature glass vials were cooled to room temperature and oxygen concentration was measured again. Statistical analysis for seed germination and head space ethanol levels in vials were performed using Genstat 14 th edition.

RESULTS AND DISCUSSION

Ethanol production of aged seeds

Ethanol production by At 362 paddy seeds aged for different periods at 75% of RH and 43 ^{0}C of temperature is illustrated in Figure 1. There is a significant difference ($P<0.001$) of ethanol production by seeds in ageing treatments. Moisture content and incubation temperature have significantly ($p<0.05$) influenced the production of ethanol. Generally a clear difference of ethanol production could be seen between paddy samples aged under different conditions. Figure 2, shows ethanol production of At 362 paddy seeds which were aged by 100% of RH and 43 ^{0}C of temperature for different time periods. The results exhibited similar trend of ethanol production as explained above. Moisture levels and temperature have influenced with a probability value of $p<0.001$ and $p<0.05$ significantly and respectively on ethanol production by seeds.

A high volume of ethanol was produced when seeds were deteriorated for more than 6 hours under the 100% RH condition than at 75% RH. Kodde *el al.* in 2012 observed as increase in ethanol production when cabbage seeds were deteriorated by hot water treatment for a period of 6 hours. Kodde *et al.* differentiated vigour of 0.5 g of seed by measuring ethanol production using modified breath analyser with the test conditions of 30% initial seed moisture content, 40 ^{0}C temperature and incubation for a period of 6 hours. However, very high amount of (about 1200 µg/l) ethanol production was observed in their experiment when seeds deteriorated with 55 ^{0}C of hot water for 60 minutes than seed deteriorated at low temperature. In the present study ethanol production was measured using 0.5 g of At 362 rice seeds aged by accelerated ageing conditions for different time periods. Half grams (0.5 g) of paddy seeds contained only few seeds compared to cabbage seeds that were used in experiments of Kodde *et al.* This could be

the reason for production of low amount of ethanol from paddy seeds in the present study.

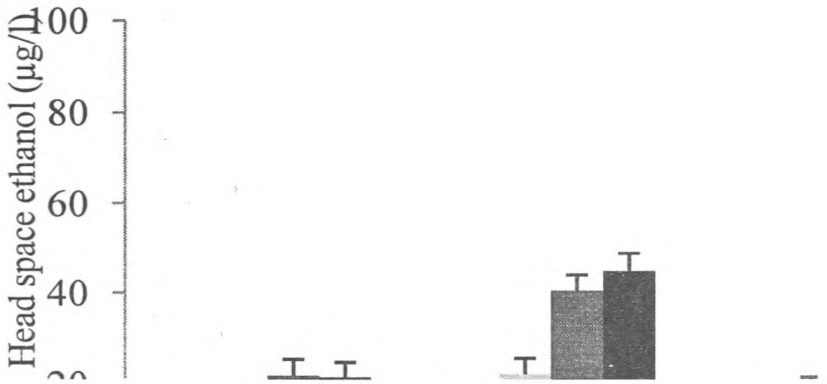

Figure 1. **Effect of seed moisture content, Incubation temperature and time for ethanol production of 0.5 g of At 362 paddy seeds which was deteriorated by 43^{0}C and 75% of RH level for different days.** Note: 40 C and 50 C represent the incubation temperature during the assay. 20%MC & 30%MC denote 20% and 30% of seed moisture content respectively, con-control, 3, 5 and 7 indicate paddy seeds samples aged at 75% RH and 43 ^{0}C for 3, 5 and 7 days, respectively.

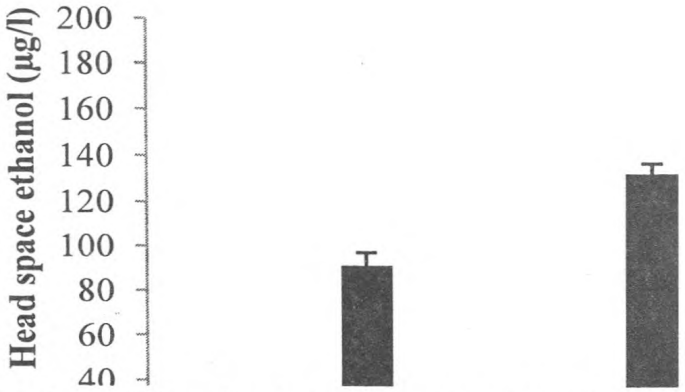

Figure 2. **Effect of seed moisture content, Incubation temperature and time for ethanol production of 0.5 g of At 362 rice seeds which was deteriorated by 43^{0}C and 100% RH level for different days.** Note: 40 C and 50 C represent the incubation temperature during the assay.20%MC & 30%MC denote 20% and 30% of seed moisture content respectively. con-control, 2, 4 and 6 indicate rice seeds samples aged at 100% RH and 43 ^{0}C for 2, 4 and 6 days respectively.

There was a significant difference ($P<0.05$) between rates of germination based on radical protrusion of different seed samples aged for different time periods. However the effect of severity of ageing treatment for total percentage (data not presented) of germination cannot be seen clearly except seed samples aged for 6 and 7 days. Rate of germination which is calculated by t_{50} value indicates the time for achieving 50% of total germination of the seed sample (data not shown). There is a significant difference ($P<0.001$) in t_{50} values of different seed samples aged for 3, 5 and 7 days and seed samples aged for 2, 4, 6 days. When the duration of ageing increases the rate of

germination has decreased and ultimately the ethanol production has increased proportional to the ageing time. The results were agreed with Kodde *et al.* (2012) as they observed delayed germination when seeds treated by hot water at 55 ^{0}C for 15 minutes. In their experiment hot water treatments for 30 minutes, the radical protrusion has decreased and the t_{50} value has increased but treated seeds for an hour have not germinated. More ethanol was produced at the incubation temperature of 50 ^{0}C than 40 ^{0}C in all treated paddy samples. The sensitivity of the ethanol assay was improved by incubating seeds over 40 ^{0}C of temperature than lower temperature (Buckly *et al.*, 2011). During anaerobic respiration, pyruvate decarboxylase converts pyruvate into ethanol (Davies *et al.*, 1974). Singer and Pensky (1952) found that the activity of enzymes was great at 40 ^{0}C than 20 ^{0}C of temperature in wheat seeds. Rice being in the group of cereals could have resulted for more ethanol production at 50 ^{0}C than 40 ^{0}C of temperature in the experiment. Although high temperature influences more volume of ethanol production, temperature over 50 ^{0}C was not recommended for the ethanol assay by Kodde *et al.* (2012) due to the occurrence of some deleterious changes by high temperature.

Out of cell degradation mechanisms, the major cause is membrane deterioration (Gholami and Golpayegani, 2011). Mitochondrial membranes are also deteriorated by oxidation either in artificial or natural seed ageing (Benamar *et al.*, 2003). In the present experiment, rice seeds were deteriorated by artificial ageing conditions. There is a positive relationship between ethanol production by rice seeds and severity of ageing condition. Therefore aged paddy seeds by natural ageing could be produced ethanol like seeds aged by artificial ageing. The oxygen level in glass vials were recorded before commencement of the ethanol assay and after the end of assay. The oxygen level never dropped below 18.5% in each treatment during the ethanol assay. There is no significant difference ($P<0.05$) in oxygen concentration initially before and after the end of assay. Seeds kept under reduced oxygen concentrations may cause anaerobic fermentation and then produce ethanol. Constant concentration of oxygen throughout the assay confirms that produced ethanol was totally due to aging not due to anaerobic fermentation.

CONCLUSIONS

Rate of deterioration of paddy seeds of variety At 362 can be differentiated using ethanol production by seeds because they produced various amounts of ethanol on the rate of seed deterioration. There is a positive relationship between ethanol level in vials measured by modified breath analyser and rate of seed germination. The level of ageing could be used as an indicator on aging and there by the germination percentage. Therefore, ethanol production with this test conditions can be used to assess seed vigour of rice seeds as an alternative test. To generalise the test conditions, testing of large number of rice verities with same test condition is recommended.

ACKNOWLEDGMENTS

Authors are grateful to Mr. Jan Kodde a member of the plant seed quality group in Plant Research International in Wageningen University, The Netherlands for his advice, assistance and help rendered in laboratory work. Sincere thanks are offered to Director/RRDI, Additional Director/RRDI, Deputy Director/Rice Research Station, Ambalantota and the staff members for providing paddy seed sample for the research work and their encouragement.

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