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RESEARCH NOTE

Optimization and sensitivity analysis of fast ethanol assay in maize seeds[1]

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ABSTRACT – The evaluation of seed deterioration is very important to control the quality of the seeds stored. This study aimed to investigate the potential of fast ethanol assay for seed quality assessment of maize stored under different conditions. The first experiment was to determine the incubating temperature, incubating time, and amount of seed used in the assay. The results showed that the best protocol for the detection of headspace ethanol was incubation of 3 g of maize seed with 20% moisture content (wet basis) in a 20 mL gas chromatography vial at 70 °C for 1.5 h. The assay induced approximately 200–700 $\mu g.L^{-1}$ of headspace ethanol, which was sufficient to identify seeds with different vigour levels. In the second experiment, the optimal conditions were used for quality assessment in aged maize seed stored for 12 months under different storage conditions. The increase in the ethanol production of stored maize seed under the controlled conditions (15 °C and 20% RH in the hermetic seal) was lower than under ambient conditions. The ethanol production levels of maize seed samples at the start of storage was significantly lesser than at six months storage ($p < 0.05$). The test limitations in deteriorated seed with different cultivars and ages will be discussed.

Index terms: mitochondrion, seed deterioration, ethanol, modified breath analyser, *Zea mays*.

Análise de sensibilidade e otimização de teste rápido de etanol em sementes de milho

RESUMO – A avaliação da deterioração de sementes é muito importante para controlar a qualidade das sementes armazenadas. Este trabalho teve como objetivo investigar o potencial de um teste rápido de etanol para a avaliação da qualidade de sementes de milho armazenadas sob diferentes condições. O primeiro experimento consistiu em determinar a temperatura de incubação, o tempo de incubação e a quantidade de sementes utilizadas no teste. Os resultados indicaram que o melhor protocolo para a detecção de etanol *headspace* foi a incubação de 3 g de sementes de milho com 20% de teor de água (base úmida) em um frasco de 20 mL para cromatografia gasosa a 70 °C por 1,5 h. O teste induziu aproximadamente 200-700 $\mu g.L^{-1}$ de etanol em *headspace*, o que foi suficiente para identificar sementes com diferentes níveis de vigor. No segundo experimento, as condições ideais foram utilizadas para avaliação da qualidade de sementes de milho envelhecidas e armazenadas por 12 meses sob diferentes condições de armazenamento. O aumento na produção de etanol das sementes de milho armazenadas sob condições controladas (temperatura de 15 °C e UR de 20%) foi menor do que nas condições ambiente. Os níveis de produção de etanol das amostras de sementes de milho no início do armazenamento foram significativamente menores que aos seis meses de armazenamento ($p < 0,05$). A limitação do teste em sementes deterioradas com diferentes cultivares e idades será discutida.

Termos para indexação: mitocôndria, deterioração de sementes, ethanol, analisador de respiração modificado, *Zea mays*.

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Introduction

Maize is the world's most important grain, based on production volume (STATISTA, 2017). It is important in upstream protein and carbohydrate food production with direct and indirect utility (such as an animal feed mixture). This cause high demand for high quality and large quantity of maize seed. Thus, fast and accurate methods for controlling seed quality are crucial in maize seed production. Especially in the tropical zone, the potential storability, or seed vigour, of maize seed is lost faster than in the temperate zone (Doijode, 2001; Harrington, 1960).

Seed longevity is important to seed producers and gene banks, so there is a need to identify seed lots with poor shelf-life. The vigour of seeds is reduced or lost during long-term storage, even when they are stored under low-temperature and low-moisture conditions, which leads to commercial and genetic losses (Yin et al., 2014).

Appropriate seed vigour testing can measure seed longevity. Seed vigour tests applied during its storage to evaluate the storability are extremely important in the seed production process. The accelerated ageing (AA) test is most often used as a vigour test for maize seeds but cannot meet the current development demands of automation and seed inventory management because of its subjectivity as the test involves evaluating seedlings and takes excessive time, approximately one week, to acquire results (Matthews et al., 2012; Nijënstein and Kruse, 2000). To solve this problem, Colville et al. (2012) proposed that volatile compounds, such as ethanol, are involved in seed deterioration and potentially identifying volatile marker compounds could be one method to diagnose seed viability loss.

Kodde et al. (2012) reported a fast and reliable method to evaluate seed viability or deterioration using a modified ethanol sensor, or breath analyser, a so-called fast ethanol assay. In a fast ethanol assay, ethanol production by partially imbibed seed is a potential indicator of seed vigour because production from deteriorated seed is relatively high compared to that from high-vigour seed (Akimoto et al., 2004; Buckley and Huang, 2011; Kataki and Taylor, 2001; Kodde et al., 2012). The generation of ethanol can indicate loss of mitochondrial membrane integrity (Brand and Nicholls, 2011; Cossins and Beevers, 1963; Logan et al., 2001). Although many studies have investigated the ethanol production of stored seed, there has been few works using large seed such as maize (Buckley and Huang, 2011; Kodde et al., 2012). The protocol optimization for fast ethanol assay in maize seed is difficult because maize seed constitutes a low amount of the living embryo per Gas Chromatography (GC) vial container volume compared to other successful species, such as cabbage (*Brassica oleracea*), cotton (*Gossypium* sp.) and lettuce (*Lactuca sativa*).

In the present paper, we report on the protocol optimization for preparing seed samples for the detection of seed deterioration using a modified ethanol sensor including investigation of the effect of natural ageing on the headspace ethanol and seed vigour in commercial maize seeds.

Material and Methods

Seed samples – Protocol optimization of fast ethanol assay in maize seed was studied with 31 samples of commercial F1 hybrid seeds (nine cultivars). Seed samples were obtained from the top-ten seed companies and seed growers in Thailand (Napasintuwong, 2015). Seeds were received without pesticide treatment. Seed samples were produced in seed production fields and then stored at 15 °C and 50% RH until used in this experiment. Seed moisture content (S.M.C.) were estimated following (ISTA, 2015) using the high constant temperature oven method. In brief, two replicates of 4.5 ± 0.5 g seed sample were dried at 130–133 °C for 1 h ± 3 minutes, and then returned to room temperature for 30 minutes. The S.M.C. was expressed as a percentage of the seed wet weight or fresh weight basis (FWB). For standard germination, seeds were evaluated using between papers techniques (ISTA, 2015) with four replications of 50 seeds each sample. The seeds in rolled paper towels were kept in polyethylene plastic bags and closed boxes and placed in a cabinet germinator (Seedburo Equipment Company, Des Plaines, USA). The RH in the germinator was maintained near to saturation. The germinator was set to 25 °C with 8 h light (40–50 $\mu mol.m^{-2}.s^{-1}$) / 16 h dark. Seeds were scored as germinated when normal seedlings occurred according to ISTA (2006). Normal seedlings were counted daily for 10 days after beginning to germinate. The percentage germination of freshly arrived seeds was more than 80%; the percentage germination did not change in seeds stored at 15 °C during the period of the experiment (Table 1). Before the experiment began, the seeds were graded to include only seeds that weighed between 250 and 350 mg and were stored at 15 °C (50% RH) in darkness prior to initiation of the experiment.

Optimal protocol for seed vigour discrimination of stored maize using fast ethanol assay – The assay was based on the headspace ethanol of the seed samples produced during heated incubation and was used to indicate seed deterioration during storage. Commercial F1 hybrid seeds of cv. A and B were divided into 2 subsamples according to the production year (2015 and 2016) and the S.M.C. for each sample was determined. Then, deionized water was added to achieve

the desired seed moisture percentage (20% S.M.C.) Kodde et al. (2012). The incubation temperatures (40 and 70 °C), incubation times (3 and 6 h) and amounts of seed per GC vial volume (3 g/20 mL and 15 g/100 mL in the GC vial) were analysed using a full factorial design. Four replications of each sample were used to measure the ethanol production using the ethanol assay as described by Kodde et al. (2012).

In brief, seeds were placed in specific GC vials. The vials were sealed with aluminium crimp caps with 3 mm-thick butyl rubber and Teflon septa immediately after adding water. The GC vials containing moistened seeds were incubated at the desired temperatures. The ethanol concentration in the headspace was measured at 3 and 6 h after incubation using a modified breath analyser (Alcotest 6810 agri; Dräger safety AG & Co. KGaA; Lübeck, GER). The modification consists in software adaptation and replacement of the mouthpiece for a syringe needle with thread. This modification allowed to measure the headspace of sealed vials or containers with other seals that can be penetrated by the needle. A disposable hypodermic needle (18 gauge, 5 cm long) was used to collect gas samples from the vials. The needle was shortened to approximately 2.5 cm to allow for pressure equilibration in the vial during its insertion. The device collected a subsample of approximately 0.3 mL from the headspace for every measurement. The analytical range of the breath analyser was 0–9,900 µg ethanol per litre of gas mixture. The breath analyser was calibrated annually by the supplier.

The optimized protocol was tested to distinguish the old and new seeds of each cultivar (A and B) and have a low standard error of the mean (*SE*). Then this protocol was fine-tuned, with cv. C, for the incubation time to improve its accuracy. After fine-tuning, the protocol was investigated regarding relationships among the head-space ethanol and AA test for the 25 seed samples by correlation analysis (Pearson correlation coefficients).

For vigour test, accelerated ageing and the single count of radicle emergence (SCRE) test was used for traditional seed vigour determination in this experiment. The AA test was carried out according to ISTA (2015). A sample of seed, one-layer deep was placed in boxes on the shelves in the ageing chamber at 42 °C. The 72 h ageing period started with the placement of the boxes in the ageing chamber. The germination test was carried out after the AA test using four 50-seed replications for each sample within 1 h after removal from the ageing chamber. The testing conditions for the tests were those outlined in the germination tests. The SCRE test was used to evaluate the vigour levels of each maize seed lot using early counts of radicle emergence (2 mm in length) according to a modified radicle emergence test described by ISTA (2015). The only difference was the incubation at 25 ± 2 °C. In this experiment, the radicle emergence count took place 40 h after setting to germinate.

Effect of natural ageing on the germination, vigour and headspace ethanol of maize seeds – Effects of natural ageing after 12 months storage in the laboratory on seed qualities and ethanol production were investigated in cv. A and B (particularly in the 2016 production year). One subsample was placed under controlled atmosphere storage that was conducted in a GrainPro® SuperGrainbag® Premium 25RZ bag and stored at 15 °C and 20% RH, whilst another subsample was placed under ambient conditions on a laboratory bench at about 30 °C and 55% RH based on monitoring using a USB data logger (Centor Thai, Bangkok, TH). At the experiment start and at 12 months, subsamples were drawn and subjected to a germination test, AA test, seed moisture content determination and analysis of ethanol production.

Data analysis – The headspace ethanol and seed moisture content analyses were carried out using analysis of variance (ANOVA fixed effect model) with completely randomized designs followed by least significant difference (LSD) post hoc tests and orthogonal contrasts to identify significant differences among means at a significance level of $p \leq 0.05$. Homogeneity of variance (Levene test) and normality of data were tested in accordance with the assumptions for ANOVA. The percentage data, such as germination, AA test, and SCRE test, were angularly transformed before the ANOVA was carried out (transformed by arcsine; untransformed values are shown in the table and figure to facilitate comparison). Correlations between AA test and headspace ethanol was calculated using R, and plotted with ggplot2.

Results and Discussion

Optimal protocol for seed vigour discrimination of stored maize using fast ethanol assay

Prior to optimizing the protocol, the seed qualities of cv. A and cv. B were determined. The seed moisture content of all samples before the experiment was ranked from 9.73 to 10.44% on a wet weight basis (Table 1). Each sample had been graded and stored at 15 °C and 50 % RH for 3–5 months before the experiment. Thus, seeds would have equilibrated to this specific moisture level. The germination levels of maize seeds of each cultivar from the different production years were not significantly different (Table 1). The germination ranges of each cultivar were approximately 98.5–100.0%.

The headspace ethanol obtained from the protocol of P8 (incubated seed at 70 °C for 6 h in a 100 mL GC vial) had the highest value (1014.50 µg L^{-1}). Preparation of seeds by incubation

at 40 °C (P1–P4) was significantly different from those of the protocol of incubation at 70 °C (P5–P8) when considered in terms of ethanol production (Table 2). However, with the preparation of seeds using incubation at 40 °C it was not possible to classify the seeds with different production years for both A and B cultivars, although this method had a low *SE* (Figure 1a–d).

Table 1. Seed moisture content and germination percentage of maize seed samples before the experiment.

Lot	Cultivar	Production year	Seed moisture content (% wet weight basis)	Germination (%)
1*	A	2015	10.03	98.5
2	A	2016	9.73	100.0
3	B	2015	10.21	98.5
4	B	2016	10.44	99.5
5	C	2015	10.38	98.50
6	C	2016	10.20	98.75
7†	D	2015	9.80	97.50
8	D	2015	10.20	98.50
9	E	2015	9.30	98.00
10	E	2015	10.00	95.00
11	F	2015	10.70	97.00
12	F	2015	10.20	96.50
13	F	2015	9.70	93.50
14	F	2015	10.20	89.50
15	F	2015	10.70	99.00
16	F	2015	10.20	99.00
17	F	2015	9.70	94.50
18	F	2015	10.20	94.50
19	G	2016	15.09	99.00
20	G	2016	15.01	90.50
21	G	2016	14.80	88.50
22	G	2016	14.72	93.00
23	G	2016	15.20	89.50
24	G	2016	14.57	93.00
25	H	2016	14.08	85.50
26	H	2016	11.58	85.00
27	H	2016	11.10	86.00
28	H	2016	11.58	84.00
29	H	2016	10.46	94.50
30	H	2016	10.87	90.50
31	I	2016	11.11	83.50

* Lot 1–2 of cultivar A, lot 3–4 of cultivar B and lot 5–6 of cultivar C were used for the protocol optimization.
† Lot 7–31 of cultivar D–I were used for correlation analysis.

Methods of incubation at 40 °C cannot stimulate maize seed to produce and release headspace ethanol to distinguish between old and new maize seeds (Figure 1a-d). In contrast, seed preparation to activate ethanol production for fast ethanol assay via incubation at 70 °C provided sufficient headspace ethanol for classification of the different seed vigours of the two cultivars (Figure 1e-h). Kreuzwieser et al. (1999) reported that the increase in the headspace ethanol was due to seed deterioration. During aerobic respiration, in functional mitochondria, glucose is converted into two pyruvate molecules and NAD^+ is reduced to NADH which is oxidized to NAD^+. When mitochondria are not functional, an alternative mechanism of regenerating NAD^+ is needed. This is provided by the conversion of pyruvate to ethanol or lactic acid during fermentation. In addition, the protocol of maize seed preparation for fast ethanol assay at 70 °C for 3 h in a 20 mL GC vial had a low *SE* (Figure 1e). This reflected the high accuracy as well as this protocol required less time and low work resources.

Thus, the protocol of incubation at 70 °C for 3 h in a 20 mL GC vial was fine-tuned using cv. C to determine a more accurate incubation time. The result of this latter experiment showed that the best protocol for the detection of the headspace ethanol was incubation of 3 g of 20% S.M.C. (FWB) maize seed in a 20 mL GC vial at 70 °C for 1.5 h because new and old seed were clearly distinguished during the test (Figure 2). Furthermore, an incubation time of 1.5 h is very short and provides an effective endpoint for fast ethanol assay in maize seed. In Figure 2, data measurements

Table 2. Effect of incubation temperature, incubation time, and container size on the headspace ethanol of maize seed (cultivar A and B).

Protocol	Means of headspace ethanol ($\mu g.L^{-1}$)
P1 (40 °C, 3 h, 20 mL)†	49.75 d*
P2 (40 °C, 3 h, 100 mL)	70.16 d
P3 (40 °C, 6 h, 20 mL)	15.06 d
P4 (40 °C, 6 h, 100 mL)	40.38 d
P5 (70 °C, 3 h, 20 mL)	518.19 c
P6 (70 °C, 3 h, 100 mL)	450.88 c
P7 (70 °C, 6 h, 20 mL)	747.38 b
P8 (70 °C, 6 h, 100 mL)	1014.50 a
p	≤ 0.01
Coefficient of variation (CV; %)	52.39

*Mean values in a column followed by the same letter are not significantly different at the probability level of 0.05.
† Amounts of seed per GC vial volume: 20 and 100 mL are 3 g and 15 g respectively.

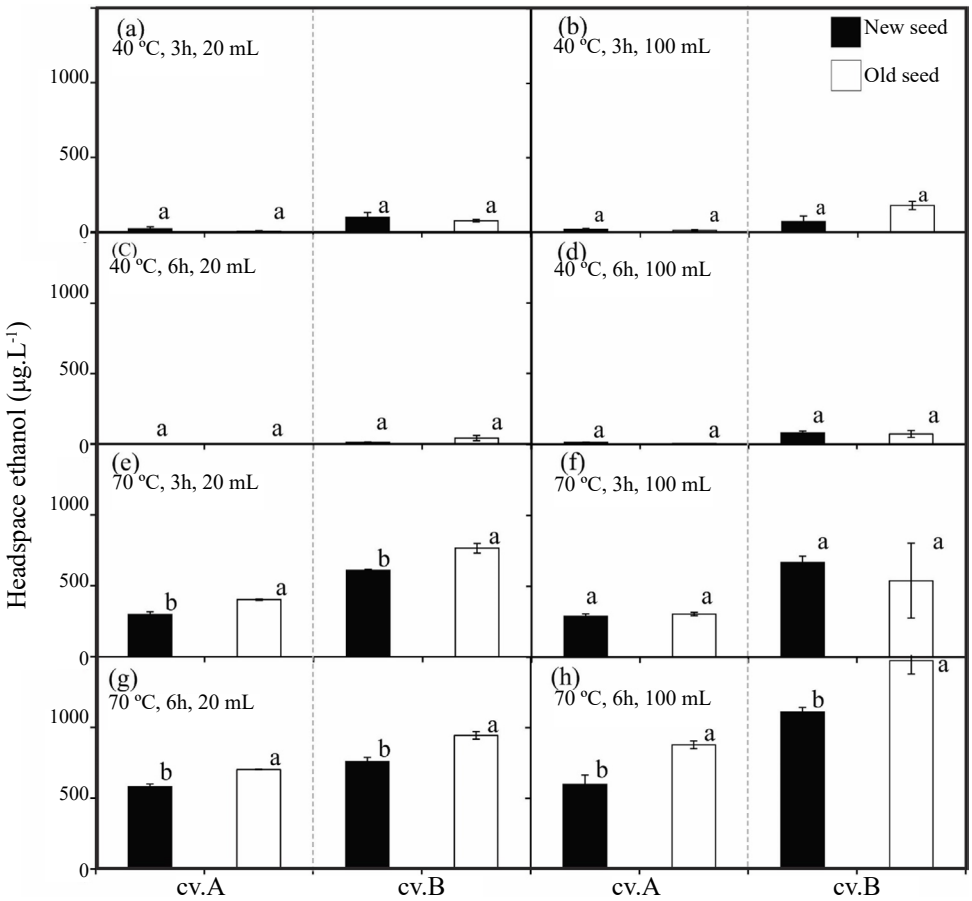

Figure 1. Headspace ethanol of F1 hybrid maize seed cv. A and B in different production years (new and aged seed) after measuring headspace ethanol using various protocols of fast ethanol assay. Amounts of seed per GC vial volume are 3 g and 15 g for 20 mL (a, c, e and g) and 100 mL (b, d, f and h), respectively. Mean values in each bar within the same cultivar followed by the same letter are not significantly different (LSD test at $\alpha = 0.05$). Error bars denote *SE* (n = 4); missing error bars indicate *SE* was smaller than could be displayed.

reveal a clear distinction for the ethanol values only for 1.5 h incubation time whereas for the other time durations the results are mixed. This phenomenon offers an understanding of optimization of fast ethanol assay for evaluation maize seed deterioration because the severe conditions, high temperature and long incubation time in this case, will disrupt old seed after released the headspace ethanol slightly but final volume equal to the ethanol produced from the new seed that is still produced and consumed ethanol efficiently. Also, it will be unable to classify the ethanol eliminatory potential of non-aged seeds from those aged seeds using conditions that cannot stimulate the seed to produce ethanol because the final volume of headspace ethanol from both of them are equal (see also incubation time at 1 h in Figure 2).

Appropriate seed vigour testing can measure seed longevity. Mitochondrial membrane degradation is a good first warning sign in seed longevity prediction for seed inventory management. The mitochondrial membrane is known to be among the primary sites of deterioration, for instance, during seed ageing, because mitochondrial activity has been clearly demonstrated to be the primary source of energy (adenosine triphosphate) for cells (Delouche and Baskin, 1973; Nelson and Cox, 2012). A decrease in the mitochondrial efficiency is correlated with an increase in the production of volatile compounds, especially ethanol (Akimoto et al., 2004; Colville et al., 2012; Mira et al., 2016; Woodstock and Taylorson, 1981). In the past, the method has been based on benchtop multiparameter instruments, using gas chromatography or enzyme sensor technology, both of which are slow and costly for routine analyses of multiple samples (Bicanic et al., 2003; Kataki and Taylor, 2001; Mira et al., 2010; Mira et al., 2016). Kodde et al. (2012) reported a simplified method of measuring seed-produced ethanol under anaerobic conditions with a modified breath analyser, a so-called fast ethanol assay.

Using a modified breath analyser for fast ethanol assay has been reported only for small seeds of dicotyledonous species, especially *Brassica*, such as cabbage (*Brassica oleracea* L.) and canola (*Brassica napus* L.), where these seeds have a high amount of living part in the seed (such as the embryonic axis and cotyledon) per volume and can produce an appropriate amount of headspace ethanol for the modified breath analysis (Buckley and Huang, 2011; Kodde et al., 2012). We tested whether maize seed ageing could also produce sufficient headspace ethanol by placing a small number (10) of seeds at 20% S.M.C. (FWB) under incubation at 70 °C for 1.5 h. Our experiments confirmed that an increase in the seed moisture content and temperature can also accelerate the production of headspace ethanol and the results showed that maize seeds with different vigour levels can be differentiated by using this method (Figure 2). Furthermore, our experiments are not influenced by microbial contamination that could produce surplus ethanol (data not shown).

The assay is an accurate and convenient seed vigour test compared to gas chromatography and tests based on germination and the TZ test, as well as detecting ethanol released from seeds using Resazurin (Brand and Nicholls, 2011; ISTA, 2015; Min, 2012). The fast ethanol assay requires 2–3 h for complete analysis. A moisture content of 20% seems optimal for determining ethanol production from maize seeds. Also, this is below the minimal moisture content (30%) required for radicle protrusion (Copeland and McDonald, 1995). For accurate information to discriminate seed vigour from the headspace ethanol, work with larger sample sets and with different storage periods is required to study the patterns of headspace ethanol.

Relationships among head-space ethanol and AA test of 25 maize F1 hybrid seed lots with different production histories were investigated with correlation analysis. A negative linear correlation was observed, with a high AA test correlating with low ethanol production and *vice versa* (r = -0.776; p = 0.0050) as shown in Figure 3.

Protocol optimization of fast ethanol assay in maize seed showed that the incubation time, incubation temperature, and ratio of seed per analytical vial were factors to enhance the differences in the headspace ethanol produced by different maize seed samples. Maize seed is large but contains a small living embryo, so the ethanol production can be quite varied when factors related to seed preparation in the assay are not adjusted correctly. Our results yielded higher Pearson correlation coefficients with the AA test (Figure 3). The results are in line with Buckley and Huang (2011) in that a fast ethanol assay is a better indicator of seed vigour than standard germination in the standard germination range of 80–100%.

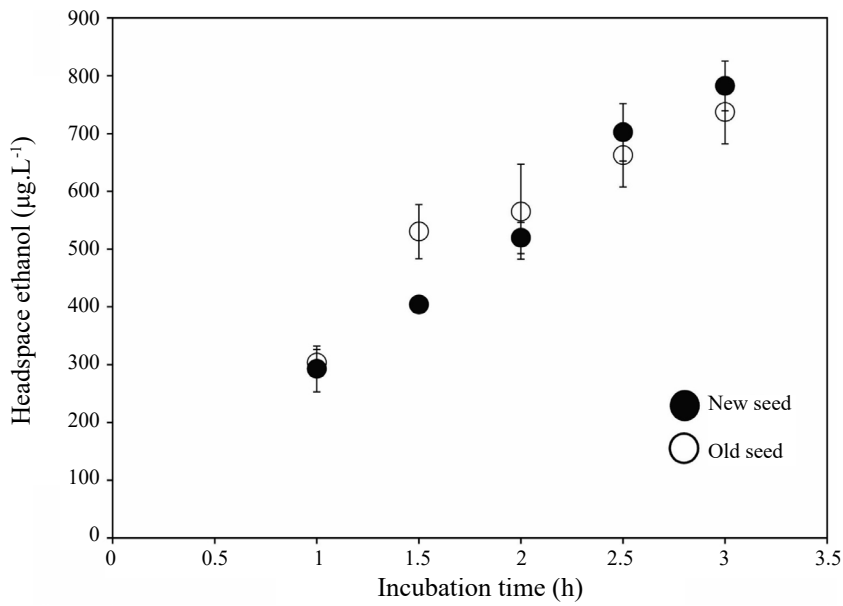

Figure 2. Headspace ethanol of F1 hybrid maize seed cv. C in different production years (new and old seed) after measuring headspace ethanol at various incubating times of fast ethanol assay at 20% seed moisture content (fresh weight basis) and 70 °C in a 20 mL gas chromatography vial. Error bars denote *SE* (n = 4); missing error bars indicate *SE* was smaller than could be displayed.

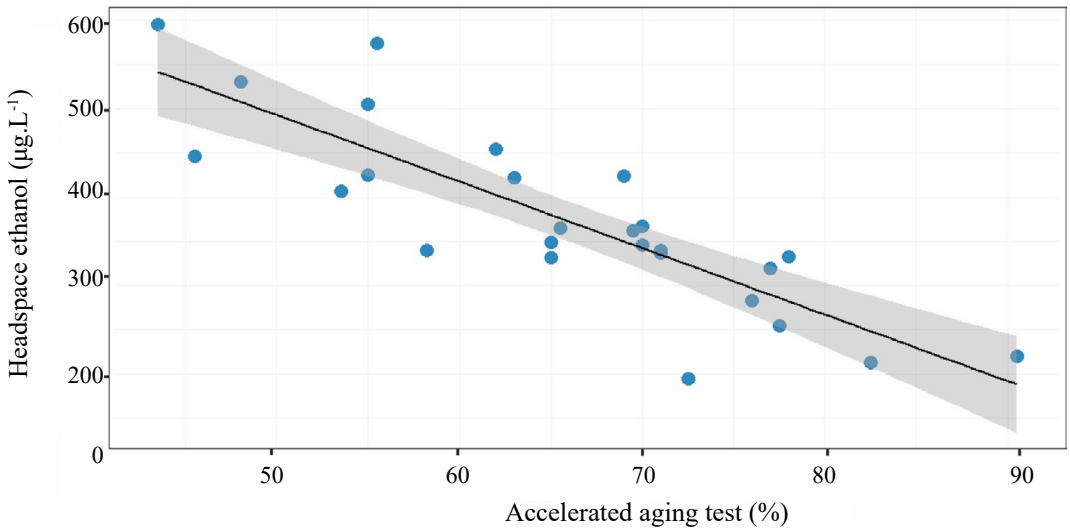

Figure 3. Relationship between headspace ethanol concentrations and the accelerated ageing test of 25 F1 hybrid maize seed samples that had standard germination of more than 80%. Linear regression line is shown (r = -0.776; p = 0.0050). Darker shaded areas indicate confidence intervals of the linear model fitted.

Effect of natural ageing on the germination, vigour and headspace ethanol of maize seeds

To verify whether the ethanol production and seed quality changes in aged maize seed after storage for 12 months under different conditions, we further studied the optimal protocol from the previous experiment. In Figure 4, the interactions between storage times, cultivars, and storage conditions were identified for various parameters.

Germination percentage did not change under the controlled atmosphere after six months storage but was significantly decreased at 12 months storage (Figure 4a–b). In contrast, seed germination decreased continuously under storage in polyethylene bag and ambient conditions on a laboratory bench (30 °C and 55% RH) (Figure 4c–d).

The AA test was used for testing seed vigour. The results of the AA test differentiated natural ageing for both cultivar A and B and could differentiate ageing in a situation where there are interactions between effect of cultivars and storage conditions (Figure 4a–d). Germination after AA test of cv. B stored under ambient was greatly reduced. This reflects the sensitivity to deterioration in ambient conditions of different maize cultivars. In AA test, high vigour seed lots will withstand these extreme stress conditions, high temperature and high relative humidity, and age more slowly than low vigour seed lots. Thus, after AA, high vigour lots retain a high germination, whilst that of low vigour lots is reduced. The AA procedure resulted in medium-to-high water activity and enzymatic activity, which would begin to protect or repair the deterioration processes occurring in seeds (Bewley et al., 2013; Groot et al., 2012). In maize seed testing, the AA test takes 10–13 days for complete analysis, whilst a determination of germination or radicle emergence speed, such as t_{50} and single count of radicle emergence test, takes 4–5 days (Matthews et al., 2011). The results revealed that both cultivars could be differentiated using the SCRE test. The difference in the results of SCRE test for seed lots of both cultivars between the control and 12 months storage was approximately 62%.

Natural ageing profiles of the headspace ethanol were clearly observed in the present investigations (Figure 4a–d). The fast ethanol assay can detect maize seed deterioration in both cultivars A and B. Regarding their storage histories, new and old seeds of both cultivars were analysed separately for 12 months. The ethanol production levels from the control (at the start of storage) and after 12 months storage of cultivars A and B were significantly different. In cultivars A and B, the headspace ethanol of the control seed lots was approximately 380 µg. L^{-1} compared to 470 µg. L^{-1} in the 12 months storage seed lots. An increase in the ethanol production of stored maize seed was found under both the controlled atmosphere conditions and the ambient conditions but the ethanol production was higher in the ambient (Figure 4a–c). This phenomenon may be due to the efficiency of hermetic storage in the GrainPro® bags. The positive effects of the cool-dry environment and hypoxia on seed storage using GrainPro® bags have been well documented (Baoua et al., 2013; García-Lara et al., 2013).

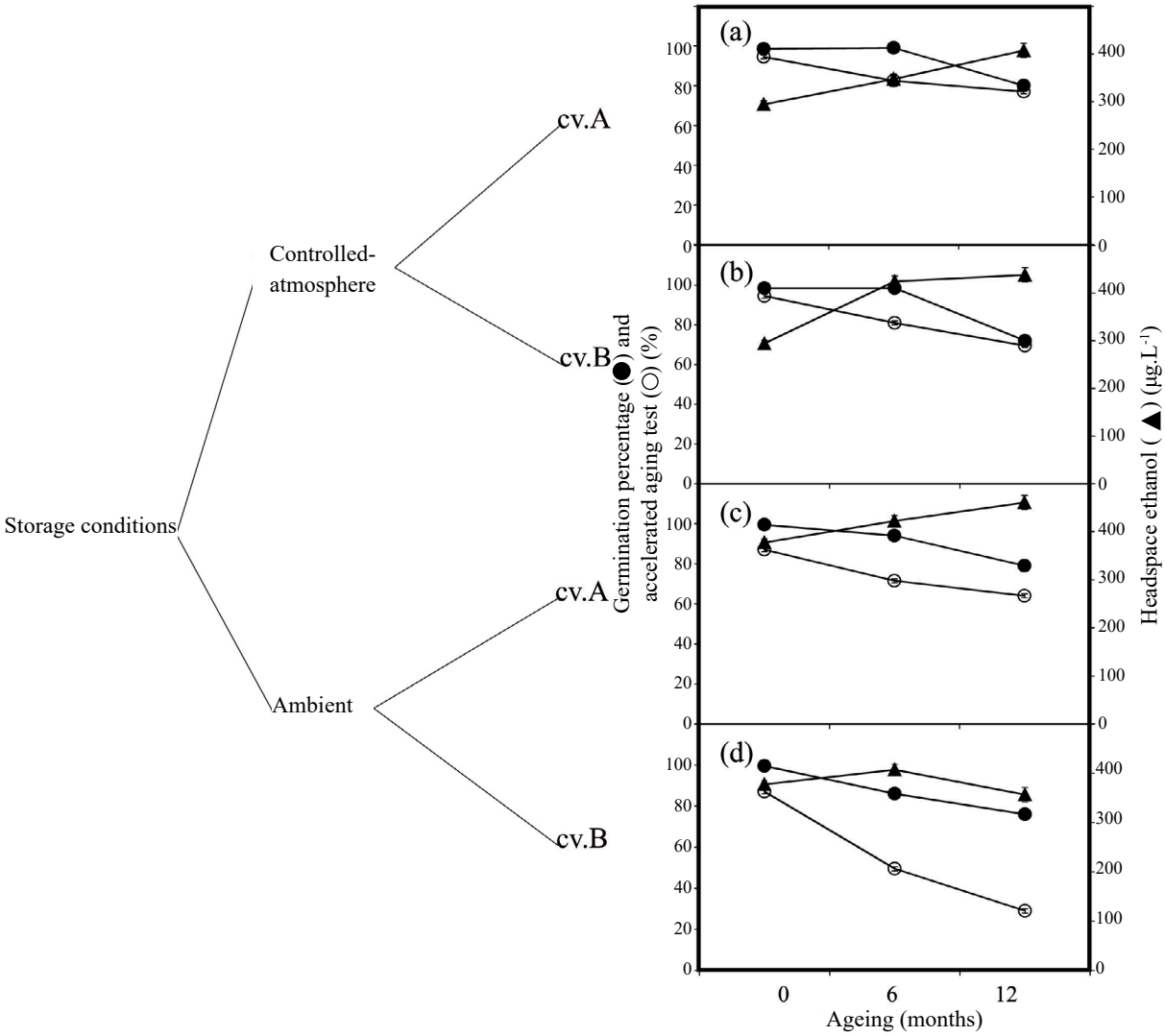

Figure 4. Seed quality of F1 hybrid maize seed cv. A (a and c) and B (b and d) after storage under different conditions (controlled atmosphere and ambient) and times (0, 6 and 12 months): germination percentage (●), accelerated ageing test (○) and headspace ethanol using the protocol of incubation of 3 g of 20% S.M.C. (FWB) maize seed in a 20 mL GC vial at 70 °C for 1.5 h (▲). Error bars denote *SE* (n = 4); missing error bars indicate *SE* was smaller than could be displayed.

The results of natural ageing profiles of the headspace ethanol in this experiment was consistent with research reported using cabbage seeds. Naturally aged cabbage seeds can be distinguished by fast ethanol assay (using 500 mg seed samples at 30% moisture content in 20 mL GC vials after 3.5 h incubation at 40 °C) after storage for 10 months at 20 °C and 40–50% RH (Kodde et al., 2012). The seed moisture percentage and incubating temperature for our assay were 20% and 70 °C, respectively. These conditions induced headspace ethanol levels of 200–700 µg. L^{-1}, which is close to the results reported in the investigation of canola (Buckley and Huang, 2011), but lower than for cabbage seed using the conditions of Kodde et al. (2012). Our results were at the same level as for aged maize seed after 11–18 days in a water bath at 45 °C (400–500 µg. L^{-1}) using a Biochemistry Analyser™ that reported by Kataki and Taylor (2001).

Maize seeds are larger than *Brassica* seeds and may have different ethanol production patterns (Thomson et al., 2005). Interestingly, our protocol, using approximately 10 seeds in a 20 mL GC vial, could produce enough headspace ethanol to determine maize seed vigour within a few hours; it might take up to 12 h, however, to perform this task using the protocol of Kataki and Taylor (2001). It is noteworthy that the variation in the headspace ethanol levels measured from four replicates

was still quite high, even though the F1 hybrid seed samples were uniformly graded to 250 and 350 mg. Consequently, more replicates should be used in future analysis. Caution is needed with the limited application of fast ethanol assay using a modified ethanol sensor in seed vigour testing. Maize genetics, storage condition and duration of storage is necessary to consider for the application of fast ethanol assay. The interactions between storage times, cultivars, and storage conditions indicated distinct ethanol production peaks corresponding to seed quality, especially with the seed vigour. In maize seed, accuracy of classification will be reduced, if the seed has a lower AA test than 60% (Figure 4d). There is no universally accepted single test for assessing the seed vigour of given seed lots because it depends on the history of the different seed lots. For this reason, multiple tests using a standardized, accurate, and convenient process should be used together to make the best assessment.

It can be considered that the tests under these conditions (20% S.M.C. (FWB) at 70 °C for 1.5 h) had different efficacy in detecting differential levels of ethanol production from naturally aged maize seed. In cultivar B, ethanol production from low vigour seeds—after storing in ambient for 12 months—was more sensitive to the protocol of the anaerobic conditions in the fast ethanol assay compared to the control and six months of storage (Figure 4d). During the storage, high-vigour (control) seeds can produce significant amounts of ethanol under anaerobic conditions (Kataki and Taylor, 2001); consequently, anaerobiosis was avoided in the earliest stage of our protocol. At the earliest stage of our protocol, the headspace ethanol of the seed sample was zero (data not shown), after which ethanol production increased by 3 g of maize seed samples in the assay. In contrast, low vigour seeds—especially in seeds of cv. B aged for 12 months in ambient— died after 1.5 h incubation at 70 °C. Seeds with higher vigour, however, were still alive (high percentage viability), although had been incubated at 70 °C for 1.5 h, in contrast to the 12 months old seed of cv. B which died quickly after beginning the assay. It could therefore produce some headspace ethanol, but the final amount was close to the ethanol production of control seed. This fluctuation may have been due to the ethanol eliminatory potential of high vigour seeds. Fluctuating artificial ageing profiles of the headspace ethanol have been reported in other studies (Buckley and Huang, 2011; Kataki and Taylor, 2001). The profiles are likely to be the net effect of the production and consumption of ethanol. In eukaryotic cells, the alcohol dehydrogenase 1 and 2 play an important role in the accumulation and reduction of ethanol, respectively (Manzo-Avalos and Saavedra-Molina, 2010; Maricle et al., 2014; Thomson et al., 2005). Seeds in general produce ethanol during germination (Rumpho and Kennedy, 1981), although high quality seed appears to reduce or eliminate ethanolic fermentation sooner than aged seed. Therefore, the optimum time and incubation temperature for the analysis of the headspace ethanol in the fast ethanol assay will occur when seeds are metabolically active until the seed dies. It is possible that the control seed had some effective mechanism for ethanol consumption that the 12 months old seeds – in ambient– did not.

Conclusions

It was possible to use the headspace ethanol produced to efficiently distinguish between low- and high-vigour seeds after natural ageing. The detection of the headspace ethanol using the protocol of incubation of 3 g of 20% S.M.C. (FWB) maize seed in a 20 mL GC vial at 70 °C for 1.5 h can distinguish new and old seed clearly. Although we sought to optimize the protocol, it is crucial to use multiple tests by comparing the fast ethanol assay with direct tests such as field emergence. In conclusion, the ethanol assay developed here may offer such a rapid assay for monitoring of maize seed deterioration in warehouse.

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