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Enhancement of Germination Rate of Aged Seeds by Ethylene

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ABSTRACT

Naturally and artificially aged seeds of rape, *Brassica napus* L., produced less ethylene than freshly harvested seed during the early stage of germination. With freshly harvested seeds one peak of ethylene production was observed during germination, which coincided with the emergence and elongation of root and cotyledon, accompanied by splitting of the seed coat. Application of exogenous ethylene was effective in accelerating germination in aged seeds but did not significantly improve the percentage of germination. Ethylene as a hormone was considered to serve as a stimulator of germination and growth. One of the factors causing seed aging might be the degeneration of an ethylene-producing system in the seed. Exogenous ethylene may be effective only for the seeds in which the ethylene-producing system is weakened but the following responding systems are still functional.

Seeds of oat (11), pea (3, 11), castor bean (16), peanut (9), and subterranean clover (4) have been shown to produce ethylene during germination, but physiological mechanisms of ethylene action still remain unknown. Moreover, it has been demonstrated that application of exogenous ethylene stimulates the germination of seeds of corn (7), unripe wheat (2, 8), lettuce (1), and nondormant peanut (9); it also breaks the dormancy of seed of various species (4, 9, 10, 18, 19).

Stewart and Freebairn (17) showed that heat-treated lettuce seed which became insensitive to gibberellic acid germinated well subsequent to treatment with exogenous ethylene. Ruge (15) showed earlier that ethylene enhanced the percentage germination of aged oat seed. In the present paper, the pattern of ethylene production by rape seedlings in the early stage of germination and the stimulation of the germination of aged seeds by exogenous ethylene were studied.

MATERIALS AND METHODS

Materials used in the experiments were the seeds of rape, *Brassica napus* L., variety Chisaya-natane, harvested in 1965 and in 1969 at the Fukushima Prefectural Agricultural Experiment Station in Japan. The seeds were stored under dry conditions until the end of November, 1969. Some of the 1965 seeds were maintained at 30 C and 74% relative humidity for various lengths of time from April 8 to November 21, 1969 to age them artificially. After treatment the artificially aged seeds were sealed in tin cans and were put with the other seed at 3 C until the experiment was started.

A germination test of the seeds was conducted with three replications (100 seeds × 3) on December 10, 1969. The blotter method at 25 C was used. The first count was on the 3rd day, and the final count was on the 7th day. The number of normal seedlings, abnormal seedlings, and slowly germinating seeds was counted.

For observing ethylene production by seedlings and effects of exogenous ethylene on aged seeds, all experiments were conducted in Erlenmeyer flasks equipped with rubber serum stoppers. To prevent mold growth, the flasks, stoppers, and seeds were sterilized at the start of the experiment. Flasks containing filter paper or agar media were autoclaved at 15 psi for 20 min. The rubber stoppers were immersed in 95% ethanol and dried under sterile conditions. The seeds were treated with 95% ethanol for 5 sec, rinsed with sterile water, sterilized with 0.1% sodium hypochlorite for 30 min, and washed three times with sterile water. Seedlings were germinated and grown under continuous fluorescent light in a room maintained at 25 C. Samples of ethylene given off from seedlings in a flask were withdrawn with a 1-ml syringe through the rubber stopper. The amount of ethylene was determined by gas chromatography, using an alumina column maintained at 50 C and a flame ionization detector. Each experiment was replicated three times.

The first two experiments were conducted to follow the trend of ethylene production during germination. In the first trial, the seeds were maintained in a closed system in 25-ml Erlenmeyer flasks containing two layers of wet filter paper. A 1-ml gas sample was withdrawn daily for 9 days from each flask for ethylene determination. In the second trial, the seeds were transferred aseptically to 125-ml Erlenmeyer flasks containing 25 ml of 1% agar. In contrast to the first trial, a 1-ml gas sample was taken every 6 or 12 hr for ethylene analysis, after which the gaseous content of the flask was flushed with fresh ethylene-free air. This trial was conducted for 120 hr.

In the third experiment, ethylene was introduced into the flask containing aged seeds to assess its effect. The concentration of ethylene in the flask was determined immediately after the injection. For the purpose of absorbing carbon dioxide and ethylene given off from the seedlings in the flask, 0.2 ml of 20% KOH and 0.25 M $Hg(ClO_4)_2$, respectively, in a small polypropylene center well, were kept inside the flask. Thus, the seeds were exposed to four types of atmospheric environment during the 20-day experiment: (*a*) control, flasks sealed with rubber stoppers, (*b*) carbon dioxide-free, (*c*) both carbon dioxide- and ethylene-free, and (*d*) carbon dioxide-free but enriched with ethylene to an initial concentration of 60 μl/liter. Germination and growth of seedlings were observed for 20 days after seeding.

The criterion of germination of aged seeds was either the emergence of the radicle or the cracking of the seed coat

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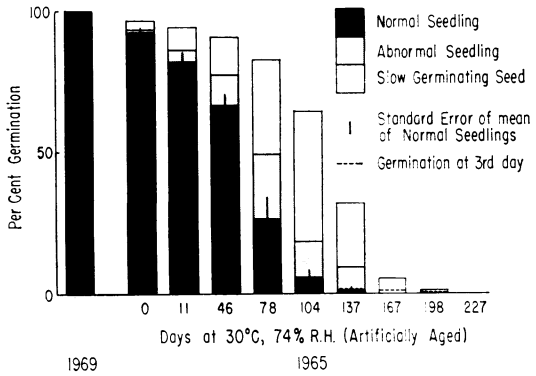

FIG. 1. Effect of artificial aging on the percentage germination of rape seed. Artificial agings were conducted by incubating the seeds at 30 C, 74% relative humidity for 0, 11, 46, 78, 104, 137, 167, 198, and 227 days, respectively. Those seeds were then germinated on blotter paper at 25 C for 7 days.

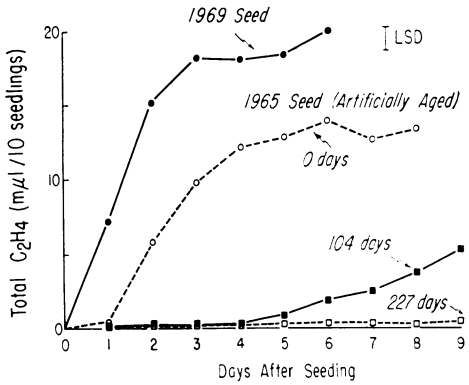

FIG. 2. Ethylene accumulation during germination of variously aged seeds of rape. This experiment was conducted in 25-ml Erlenmeyer flasks containing 20 seeds on two layers of wet filter paper. The flasks were kept closed for the entire period. 1969: new seed; 1965: seed artificially aged for 0, 104, and 227 days, respectively.

caused by the expanding cotyledon. The latter criterion was needed because some of the aged seeds started to germinate before their radicles emerged. The data on germination parameters were transformed to arc sine, according to the table of Mosteller and Yountz (12) and treated statistically by analysis of variance.

RESULTS

The percentage germination of the seeds is shown in Figure 1. All seeds harvested in 1969 germinated and became normal seedlings. Seeds harvested in 1965 and naturally aged showed slight deterioration in percentage germination; those artificially aged showed a progressive loss of viability corresponding to the length of the treatment interval.

Cumulative curves of ethylene evolution by freshly harvested and variously aged seeds during the course of their germination under a closed sterile condition are shown in Figure 2. The evolution of ethylene by new seeds (1969) was most rapid but ceased 3 days after seeding. With the naturally aged seeds (artificially aged, 0 day), the evolution was delayed 1 day, after which ethylene evolved at a rate slightly less than that of new seeds until the 5th or 6th day after seeding. With those artificially aged for 104 days, ethylene was not detectable during the first 4 days after seeding, after which it evolved very slowly until the 9th day, when the experiment terminated. Those seeds artificially aged for 227 days showed no sign of ethylene production during the experiment. These data show (*a*) that ethylene was produced endogenously by seed during the early stages of germination, and (*b*) that there is a correlation between the viability and vitality of the seeds and their ability to produce ethylene.

Figure 3 shows the rate of ethylene production at the various stages of germination by fresh and aged seeds when the internal atmosphere was flushed at 6- or 12-hr intervals. Ethylene production by new seeds was not detectable during the initial 12 hr after seeding; it began to increase between the 12th and 18th hrs, reaching a maximal rate between the 30th and 36th hrs after seeding. This peak of ethylene production corresponded to the time of radicle elongation, cotyledon expansion, and splitting off the seed coat. Subsequently, the rate decreased sharply until the 48th hr, after which it became constant at approximately 0.2 mμl per hr per 10 seedlings. With old seeds artificially aged for 11 days, ethylene became detectable after 30 hr. A small, flat peak was observed at 54 to 60 hr after seeding, which then declined slowly. The aged seed did not germinate simultaneously, which may account for the flatness of the peak and the slow decline in ethylene evolution. With seeds aged artificially for 46 days, ethylene was produced in detectable quantity 36 hr after seeding, gradually increasing to a constant rate of 0.2 mμl per hr per 10 seedlings after the 60th hr.

In fresh and aged seeds, the beginning of ethylene production corresponded with the start of germination (radicle emergence). The maximal rate of ethylene production in fresh seed was higher than that of aged seeds.

Figure 4 shows the effect of the atmospheric composition on the germination of variously aged seeds. Mean values of ethylene concentration in the control flasks (treatment 1) containing seeds artificially aged 104, 137, 167, 198, and 227 days were 0.344, 0.334, 0.298, 0.256, and 0.084 μl/liter, respectively, at the end of the experiment. The values for the carbon dioxide-free treatment were similar to those of the

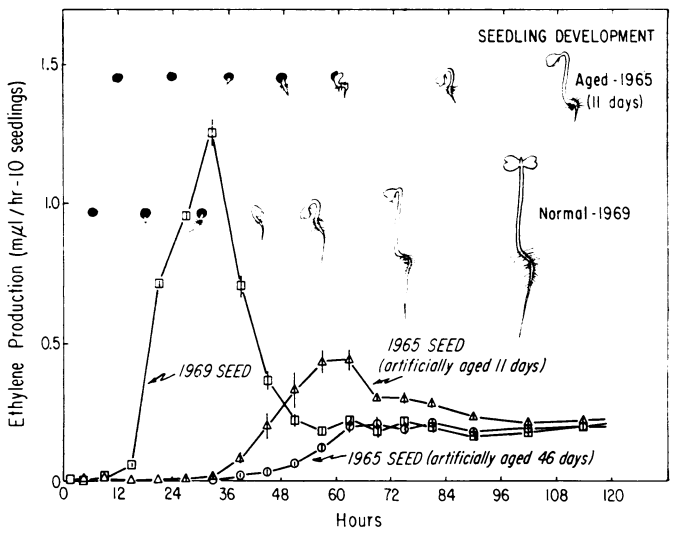

FIG. 3. Ethylene production of rape seedlings during the early stages of germination in new and aged seeds. This experiment was conducted in 125-ml Erlenmeyer flasks containing 50 seeds on 25 ml of 1% agar medium without nutrients. Each point shows the mean of triplicates with standard error. The air in the flask was replaced after every measurement, and CO_2 produced by seeds and seedlings was trapped by 20% KOH. Seedlings in the figure indicate approximate stages of germination.

control. In the carbon dioxide- and ethylene-free treatment, there was no ethylene in the flask. In treatment 4, the ethylene concentration was 60 μl/liter at the beginning of the experiment, but it decreased to values ranging between 28 and 35 μl/liter by the end of the experiment; however, the mean value at the end of the 7th day was still about 43 μl/liter. Table I shows the statistical evaluation of the germination percentage between treatments by seed age and days after treatment.

The germination rate in the ethylene treatment was always higher than in the other atmospheric treatments during the early stage of germination, except for those seeds which were artificially aged for 227 days. In this case, the percentage germination was so poor in all atmospheric conditions that significant differences could not be established. The rapidity at which seeds responded to the ethylene treatment seemed to be proportional to their age (Table I). The effect on germination of the treatments with the carbon dioxide-free atmosphere and with the carbon dioxide- and ethylene-free atmosphere was not clear. But there was a trend that these treatments slowed germination in the early stage, *i.e.*, the 5th and 6th days in the seeds artificially aged for 137 and 167 days, respectively. Seedlings seemed to develop faster with the ethylene treatment and responded with repressed elongation, thickened hypocotyl, crooked or helical hypocotyl just below the cotyledon, and inhibited root growth. But these malformed seedlings recovered when ethylene was removed.

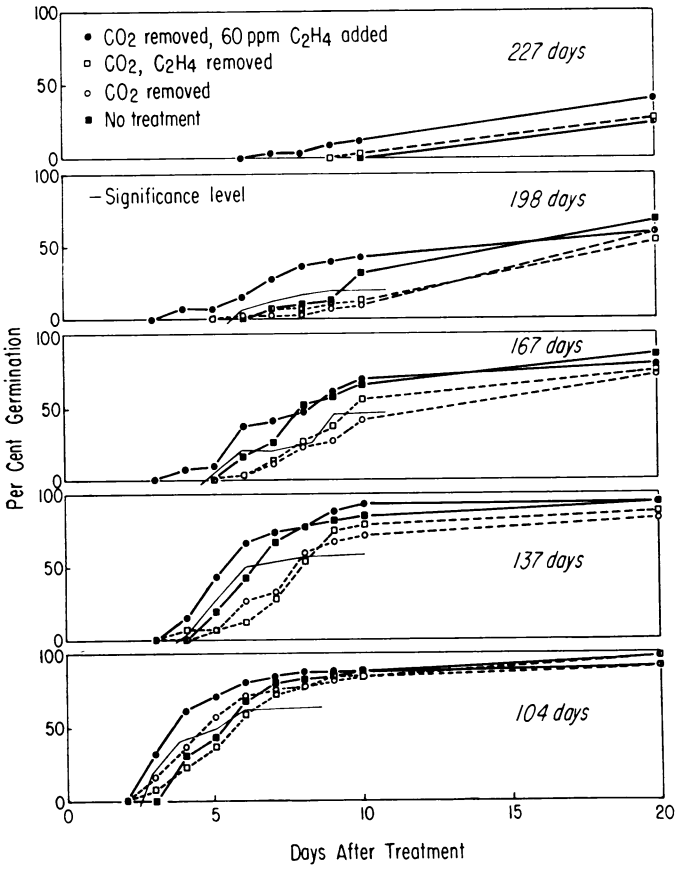

FIG. 4. Percentage germination of artificially aged seeds successively more severely aged for 104 to 227 days at 30 C, 74% relative humidity. Each seed sample (10 seeds) was germinated on an agar medium (25 ml) enriched with White's (20) nutrient solution in 125-ml Erlenmeyer flasks, with three replications and four different atmospheric conditions: ■ = no treatment (sealed with a rubber stopper), ○ = carbon dioxide removed, □ = both carbon dioxide and ethylene removed, and ● = carbon dioxide removed and ethylene (60 μl/liter) added. All flasks were kept closed for the entire period. The points (■, □, and ○) below the thin line are significantly lower (percentage germination) than the treatment with ethylene added (●) (5% level).

Table I. *Comparisons of Significances of Germination Percentages (Fig. 4) between Treatments of Variously Aged Rape Seeds with Germination Time*

Age of Seed	Comparison between Treatments[1]	Days after Treatment[2]								
		3	4	5	6	7	8	9	10	20
days										
227	2 to 1									0
	3 to 1									0
	3 to 2									0
	4 to 1					0	0	0	0	0
	4 to 2					0	0	0	0	0
	4 to 3					0	0	0	0	0
198	2 to 1				0	0	0	0	−	0
	3 to 1				0	0	0	0	0	0
	3 to 2				0	0	0	0	0	0
	4 to 1		0	0	+	+	+	+	0	0
	4 to 2		0	0	+	+	+	+	+	0
	4 to 3		0	0	+	+	+	+	+	0
167	2 to 1				−	0	0	0	0	0
	3 to 1				−	0	0	0	0	0
	3 to 2				0	0	0	0	0	0
	4 to 1		0	+	+	0	0	0	0	0
	4 to 2		0	+	+	+	+	+	+	0
	4 to 3		0	+	+	+	0	+	0	0
137	2 to 1			−	0	0	0	0	0	0
	3 to 1		0	−	−	0	0	0	0	0
	3 to 2		0	0	0	0	0	0	0	0
	4 to 1		+	+	+	0	0	0	0	0
	4 to 2		+	+	+	+	0	0	0	0
	4 to 3		0	+	+	+	+	0	0	0
104	2 to 1	+	0	0	0	0	0	0	0	0
	3 to 1	+	0	0	0	0	0	0	0	0
	3 to 2	−	0	0	0	0	0	0	0	0
	4 to 1	+	+	+	0	0	0	0	0	0
	4 to 2	+	+	0	0	0	0	0	0	0
	4 to 3	+	+	+	+	0	0	0	0	0

[1] Treatment 1 is control, 2 is CO_2 removed, 3 is both CO_2 and C_2H_4 removed, and 4 is CO_2 removed and 60 μl/liter C_2H_4 added.

[2] + indicates significantly higher germination percentage (5% level). − indicates significantly lower germination percentage (5% level). 0 indicates no significance. Blank spaces indicate no germination. Example: For 104-day aged seed, comparison of 4 to 3 after 3 days of germination, the germination percentage of treatment 4 was significantly higher than that of treatment 3.

DISCUSSION

Ethylene Production during Germination. Freshly harvested rape seeds showed a single peak of ethylene production in the early stage of germination (Fig. 3). The results were the same whether the seeds were germinated in rubber-sealed flasks (Fig. 2) or in flasks flushed periodically with ethylene-free air (Fig. 3). However, with castor beans, Spencer and Olson (16) observed that there were three peaks of ethylene evolution

during germination. These peaks corresponded with (*a*) cracking of the seed coat 3 days after seeding, (*b*) hypocotyl elongation 6 to 7 days later, and (*c*) the separation of the first leaves from the cotyledon on the 15th day after seeding.

Goeschl *et al.* (6) postulated that the evolution of ethylene was in response to some physical stress and based their assumption on their observations that physical obstruction to elongation of the pea hypocotyl resulted in increased evolution of ethylene, followed by reduced length and increased diameter of both the internodes and the cells of the internodes. Burg and Burg (3) compared this phenomenon with ethylene evolution during germination of peas and assumed that physical stress is not a primary cause of ethylene production because, after radicle emergence, the ethylene production continued and remained constant while the epicotyl expanded.

In contrast to peas, rape seeds showed a single peak. The beginning of ethylene emanation from the seed coincided with the emergence of the radicle. The maximal rate was at the stage of root elongation and cotyledon expansion, and subsequently the rate decreased sharply. The hypocotyls of 54- to 80-hr-old seedlings (Fig. 3) crooked in a way similar to the plumular hook of pea seedlings (5), and after that the hypocotyls became straightened and the cotyledons opened upward. The advanced straightening of the hypocotyls was observed in seedlings grown in ethylene-free atmosphere. Exogenous ethylene had no harmful effects on germinating rape seeds during the first 48 hr after seeding. When exposed beyond 48 hr, root and hypocotyl elongation was inhibited, and thickening of the hypocotyls and crooked or helical hypocotyls were observed.

Seedlings with crooked hypocotyls have often been observed under field conditions where the soil surface was crusted. It has been speculated that this morphological aberration is caused by accumulation of ethylene. No experimental evidence exists which demonstrates whether the ethylene was responsible. No experimental evidence indicates whether ethylene was residual from the germination phase or whether it was produced *de novo* by the seedling in response to physical resistance of the soil.

From these observations it is assumed that endogenous ethylene may have an important physiological role in hastening the processes which follow ethylene evolution, and, therefore, seed germination.

Effects of Exogenous Ethylene on Aged Seeds. Aged seeds produce less ethylene than do new seeds (Figs. 2 and 3). Ethylene-treated (60 μl/liter) seeds started to germinate more quickly than the nontreated ones in variously aged seeds, although the percentage germination of those seeds was not significantly improved (Fig. 4). The seeds unable to produce enough ethylene responded to exogenous ethylene and started to germinate fast. It might be postulated from these findings that degradation of the ethylene-producing system or insufficiency of the substrate(s) which normally give rise to ethylene may occur in aged seeds.

Auxins (3) and gibberellic acid (10, 17) are known to stimulate ethylene formation in plant tissues, but in our preliminary observation neither 5×10^{-6} M gibberellic acid nor 1×10^{-5} M IAA accelerated germination of aged seeds. In this study, however, the exposure of aged seeds to the gas enhanced their germination and growth. Ethrel (2-chloroethylphosphonic acid, 100 μl/liter) also enhanced both the evolution of ethylene and germination of aged seeds (unpublished data). It appears that ethylene acts as an inducer or activator of enzymes, probably of mitochondrial origin (13), or hormones (3, 14).

We could speculate that there are several biochemical steps during germination. One of them may be an ethylene-producing system. Ethylene may stimulate the following steps, which might be synthesis of enzymes or hormones, or activation systems. In the aging process of seeds, the ethylene-producing system may be more sensitive (17) to adverse conditions than the following systems and may degenerate first. More advanced aging may affect both systems severely. Ruge (15) reported that exogenous ethylene increased the percentage germination of aged oat seeds. But in rape seed, ethylene seems to improve the early percentage germination, not the final percentage germination, under sterile and nutritious conditions. Exogenous ethylene can be effective only in seeds in which the ethylene-producing system is weakened but the following systems are still functional. The seeds in which the latter systems are also damaged by aging cannot respond to ethylene and fail to germinate.

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