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SHORT COMMUNICATION

Study of ethylene kinetics during and after germination of sugar beet (*Beta vulgaris* L.) seeds and fruits

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Abstract

The interaction between ethylene production and seed germination of sugar beet (*Beta vulgaris* L.) was studied. For intact fruits, deoperculated fruits and true seeds, ethylene was only produced after the start of radicle emergence. Removal of the operculum or the whole pericarp, likely allowing better water uptake and gas exchange by the true seed, actually increased the time span between the start of radicle emergence and the beginning of ethylene production compared to intact fruits. ACC (1-aminocyclopropane-1-carboxylic acid), AOA (aminooxyacetic acid), AIB (2-amino isobutyric acid) and STS (silver thiosulphate) in the imbibition medium did not influence the germination pattern. Based on these findings, the function of ethylene during the germination of sugar beet is uncertain.

Keywords: ethylene, germination, pericarp, sugar beet

Introduction

Sugar beet (*Beta vulgaris* L.) yields 30% of world sugar production (Food and Agriculture Organization, 2012). Moreover, there is growing interest in using sugar beet as an energy crop (Panella, 2011). Excellent seed germination and seedling emergence is of crucial importance for optimal field production. To improve seed and seedling performance, seed processing (cleaning, polishing and sizing), priming, pelleting and coating are applied to sugar beet fruits.

Despite the use of mutants and inhibitors of ethylene biosynthesis and action, the role of ethylene during germination remains controversial (Matilla, 2000). In many species, germination runs parallel with ethylene production, i.e. *Cicer arietinum* (Gallardo *et al.*, 1994; Gómez-Jiménez *et al.*, 2001), *Lactuca sativa* (Saini *et al.*, 1989), *Oryza sativa* (Gianinetti *et al.*, 2007) and *Pisum sativum* (Gorecki *et al.*, 1991). For some species ethylene production is considered as a cause of germination, i.e. *Amaranthus caudatus* (Kepczynski and Karssen, 1985), *C. arietinum* (Gallardo *et al.*, 1994), *L. sativa* (Abeles, 1986) and *Nicotiana tabacum* (Leubner-Metzger *et al.*, 1998), while in other species ethylene is a consequence of germination, i.e. *Arachis hypogea* (Hoffman *et al.*, 1983), *O. sativa* (Gianinetti *et al.*, 2007), *Phaseolus vulgaris* (De Proft, 1983) and *P. sativum* (Petruzzelli *et al.*, 1999). In *Arabidopsis*, ethylene affects radicle emergence by decreasing the abscisic acid (ABA) responsiveness (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000).

In sugar beet, the true seed is surrounded by a protective layer, the pericarp, which consists of a fruit cap (the operculum) and a fruit cavity. The seed surrounded by the pericarp is, in a biological sense, a fruit, referred to as the achene, i.e. the sugar beet dispersal unit (Richard *et al.*, 1989; Hermann *et al.*, 2007). The seed itself is surrounded by an inner and outer seed coat (testa). The germination process starts with water imbibition and ends with radicle protrusion through the seed coat (Bewley and Black, 1994). In order to obtain better seed quality, commercial sugar beet hybrids are polished, a process by which the soft outer part of the pericarp is removed. Coumans *et al.* (1976) noted 92% germination of true sugar beet seeds (no pericarp present), while fruits without operculum and unpolished fruits had a germination of only 73 and 25%, respectively. The inhibiting effect of the pericarp on germination has several causes. The pericarp is a physical barrier for water and oxygen uptake (Perry and Harrison, 1974; Lexander, 1981).

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Furthermore, the pericarp is a source of phenols and salts, compounds that are known to create hypoxia (Coumans *et al.*, 1976; Richard *et al.*, 1989). The pericarp is also assumed to be a highly selective barrier for the exchange of ACC (1-aminocyclopropane-1-carboxylic acid) and ABA (Hermann *et al.*, 2007).

Seed quality is a complex trait for improvement by plant breeding. Potential seed vigour markers in sugar beet have been reported, such as enzymes of lipid and starch mobilization (i.e. isocitrate lyase, α-glucosidase), protein synthesis (i.e. elongation factors), the methyl cycle (i.e. S-adenosylmethionine synthetase) and ABA-signalling (i.e. protein phosphatase 2A) (de los Reyes *et al.*, 2003; Catusse *et al.*, 2011). Ethylene production is a good indicator for seed vigour in several species such as lettuce, cabbage, tomato, snap bean, sweet corn and sunflower (Khan, 1994; Chojnowski *et al.*, 1997; Siriwitayawan *et al.*, 2003). This study investigated the kinetics of ethylene evolution as a function of germination to see if ethylene was required for germination, since ethylene has been implicated in the germination process of sugar beet (Hermann *et al.*, 2007).

Materials and methods

Plant material and seed germination

Diploid monogerm sugar beet (*Beta vulgaris* L.) fruits of one seed lot (LZD-2386) were obtained from SESVanderHave N.V. (Tienen, Belgium). These fruits were produced in 2010 in France (Nérac) and stored in paper bins at room temperature and a relative humidity of 35% until use.

All fruits used were polished. The experiments were performed on fruits, fruits without operculum ('deoperculated fruits') and true seeds. Deoperculated fruits were obtained by exerting pressure on the intact fruit with a pair of tongs. As a consequence, the operculum lifted and could be removed. True seeds were carefully isolated from deoperculated fruits using a dissection needle. Experiments were only executed with undamaged fruits/seeds.

For germination experiments, independent triplicates of 100 fruits or triplicates of 25 true seeds were incubated in the dark at 20°C in polystyrene Petri dishes (90 mm), containing one layer of moist filter paper (Whatman No. 1; 1.5 ml deionized water). Each Petri dish contained 25 fruits/seeds. Where indicated, 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma-Aldrich, St. Louis, Missouri, USA), 2-amino isobutyric acid (AIB; Acros, Geel, Belgium), aminooxyacetic acid (AOA; Sigma-Aldrich) or silver thiosulphate (STS; Sigma-Aldrich) was added to the imbibition medium. Silver thiosulphate was prepared as described by Reid *et al.* (1980). At specific time intervals, germination was counted. Radicle protrusion of both seed coats was used as the criterion for germination. To compare germination curves with each other, a Gompertz function ($y = a^*$) was fitted. From these fittings, the t_{10}, t_{25}, t_{50} and t_{75} values were derived, which indicate the time to reach 10%, 25%, 50% and 75% germination, respectively. The Gompertz function was constructed with the statistical package R, version 2.12.2 (http://www.r-project.org/), and the mean times were compared to each other with Tukey's multiple comparison test (significance level = 0.05).

Ethylene measurements

At specific time intervals, sugar beet fruits (5 times 20 fruits), deoperculated fruits (3 times 20 fruits) or true seeds (3 times 20 seeds) were carefully removed from each Petri dish and incubated at 20°C in a gas-tight 10-ml glass flask sealed with a septum (natural rubber). All repeats were measured independently. The ethylene content was measured after a incubation for 1 h by taking a 1 ml sample of the headspace. A calibration gas mix containing 1 ppm ethylene was used for the determination of the ethylene production rate. Ethylene concentrations were determined via gas chromatography (Shimadzu GC-2014; Shimadzu, 's-Hertogenbosch, The Netherlands) using a packed column (Porapak R 50/80 mesh, length 3 m, outer diameter 1/8 inch) and a flame ionization detector. The injector, the column and the detector had temperatures of 150, 90 and 250°C, respectively.

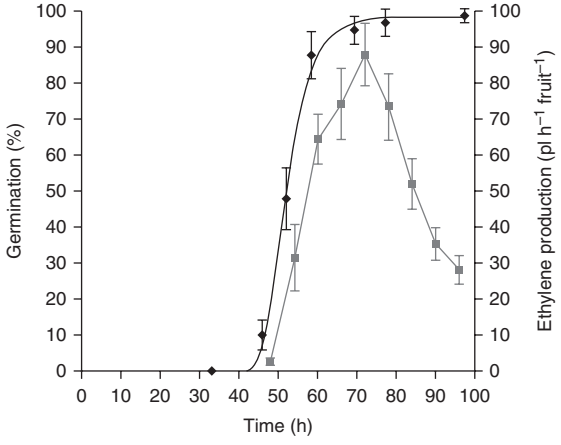

Figure 1. Germination percentage (black diamonds) and ethylene production in pl h^{-1} $fruit^{-1}$ (grey squares) of intact *Beta vulgaris* fruits. For the germination experiment each point represents the mean of three independent replicates of 100 fruits. The line represents the fitted values by means of a Gompertz curve ($R^2 = 0.997$). For the ethylene production, each point represents the mean of five independent replicates of 20 fruits. Mean values ± SD are presented.

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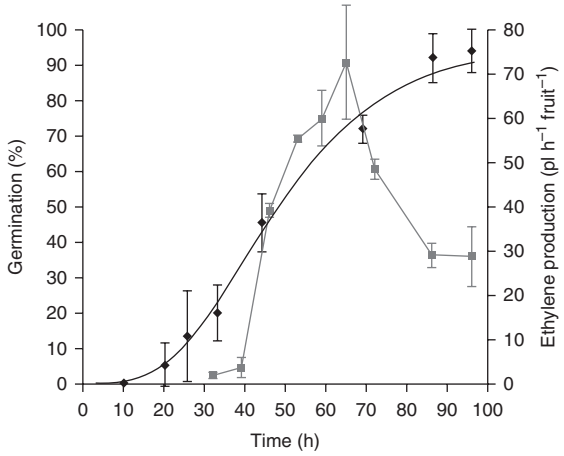

Figure 2. Germination percentage (black diamonds) and ethylene production in pl h^{-1} fruit^{-1} (grey squares) of deoperculated *Beta vulgaris* fruits. For the germination experiment each point represents the mean of three independent replicates of 100 fruits. The line represents the fitted values by means of a Gompertz curve ($R^2 = 0.995$). For the ethylene production, each point represents the mean of three independent replicates of 20 fruits. Mean values ± SD are presented.

Results

Germination and ethylene production

Germination preceded ethylene release in polished sugar beet fruits. Ethylene production began approximately 3 h after germination was first observed (Fig. 1). The first fruits germinated after 45 h of imbibition, while ethylene production was only detected after 48 h. Germination was complete 72 h after the start of imbibition. At the same time, ethylene production reached its maximal value (88 pl h^{-1} fruit^{-1}) and declined subsequently.

Removing the operculum shifted both germination and ethylene kinetics (Fig. 2). Without the operculum, germination started after 20 h, compared to 45 h with intact fruits. Ethylene production started after 32 h, which was 16 h earlier compared to intact fruits (Fig. 1). Maximal ethylene production (73 pl h^{-1} fruit^{-1}) occurred 65 h after the start of imbibition and was not significantly different compared to the maximal ethylene production of intact fruits ($P = 0.0812$).

Germination of true seeds started 8 h after the start of imbibition, whereas ethylene production was only detectable after 30 h (Fig. 3). Maximal ethylene production (68 pl h^{-1} seed^{-1}) was detected after 66 h. This production was comparable with the production obtained with deoperculated fruits ($P = 0.607$), but was significantly lower than the maximal ethylene production of intact fruits ($P = 0.0172$).

Early seedling growth and ethylene production

To determine the onset of ethylene production by the fruit, ethylene measurements were executed on well-defined stages during germination and seedling outgrowth (Fig. 4). When the operculum was not lifted, no ethylene could be detected; fruits with a lifted operculum prior to germination did not release ethylene. Ethylene was only detected once the germination of fruits started and this release gradually increased when the roots developed. Although the ethylene increase is partly due to the weight gain of the developing roots, the same pattern was obtained when the ethylene production was expressed per gram fresh weight, although less pronounced (data not shown).

Application of ethylene precursor and inhibitors

Supplementing the imbibition medium with ACC or ethylene inhibitors influenced the ethylene production of intact fruits (Table 1). Ethylene production was evaluated 72 h after the start of imbibition since this coincided with maximal ethylene release of intact fruits (Fig. 1). AIB, an inhibitor of ACC-oxidase (ACO), resulted in a slight decrease in ethylene production, whereas with AOA, an inhibitor of ACC-synthase, ethylene production was more than halved compared to the control. Further reducing the ethylene production by increasing AIB and AOA concentrations (2–5 mM), was unsuccessful since these levels were proven to be toxic (aberrant seedling growth). When the ethylene signalling was blocked with 1 mM STS, the ethylene production increased more than 50%

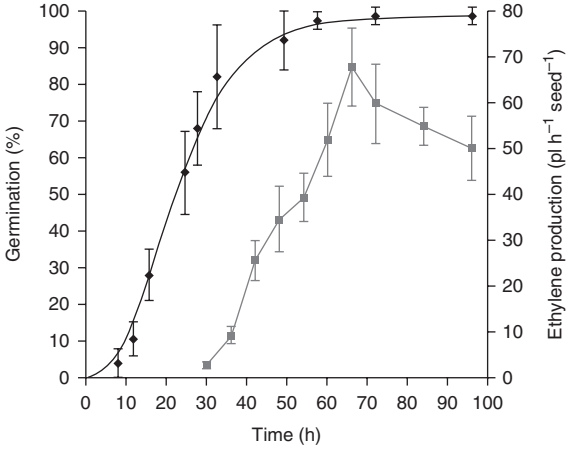

Figure 3. Germination percentage (black diamonds) and ethylene production in pl h^{-1} seed^{-1} (grey squares) of *Beta vulgaris* seeds. For the germination experiment each point represents the mean of three independent replicates of 25 seeds. The line represents the fitted values by means of a Gompertz curve ($R^2 = 0.986$). For the ethylene production, each point represents the mean of three independent replicates of 20 seeds. Mean values ± SD are presented.

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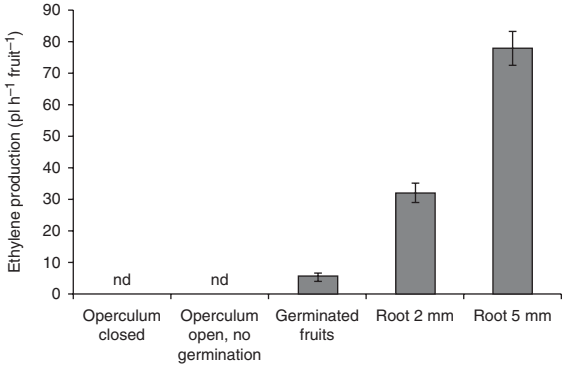

Figure 4. Ethylene production in pl h^{-1} fruit^{-1} at several stages during seedling growth. Each bar represents the mean of five independent repeats of 20 fruits. Mean values ± SD are presented; nd, not detectable.

compared to the control. Addition of 1 mM ACC to the imbibition medium resulted in a strong stimulated ethylene production of almost eight times the control value. Addition of ACC and the ethylene inhibitors had no significant influence on the germination kinetics of the fruits (data not shown).

Discussion

Intact sugar beet fruits have a restricted water and oxygen uptake compared to fruits without operculum (Perry and Harrison, 1974). Removal of the operculum leads to less mechanical resistance for the radicle to emerge and offers improved gas and water exchange. As a result, intact and deoperculated fruits show different temporal patterns of radicle emergence (Figs 1 and 2). In contrast to the findings of Hermann *et al.* (2007), seeds and deoperculated fruits also had different germination behaviour.

Like many other species, germination and ethylene production run parallel to each other in sugar beet fruits (Fig. 1). However, ethylene was only detectable just after protrusion of the radicle (Figs 1–4), suggesting that ethylene is merely a consequence of the germination process. Also, Hermann *et al.* (2007) measured ethylene evolution during germination of sugar beet fruits and found no ethylene production at t_{50}, possibly because of a lack of oxygen required for ACO. In seeds and deoperculated fruits, oxygen uptake was not a limiting factor for ethylene production (Coumans *et al.*, 1976; Richard *et al.*, 1989), which resulted in an earlier onset of ethylene production compared to intact fruits. Despite the strong induction of the ACO-transcript levels in true seed at t_{50} (Hermann *et al.*, 2007), ethylene production in true seeds is only detectable a few hours after t_{50} (Fig. 3), suggesting that ACC may be limiting at that time. This finding is supported by the results of Hermann *et al.* (2007), since they measured minimal ACC content in seeds at t_{50}. Nevertheless, removal of the operculum or the whole pericarp accelerates radicle emergence more than ethylene production. As a consequence, with true seeds the time span between the start of germination and ethylene release increases (22 h).

An unchanged germination pattern was observed after treatment with ACC, AIB and AOA, indicating that enhancing and reducing ethylene production has no influence on the germination of sugar beet fruits. Also, blocking the ethylene signalling with STS had no influence on the germination pattern, but resulted in an increased ethylene production, indicating a negative feedback control of ethylene production in sugar beet, as already reported in banana fruit, etiolated pea stems and citrus peel (Vendrell and McGlasson, 1971; Saltveit and Dilley, 1978; Riov and Yang, 1982). In addition, ethylene only becomes detectable after radicle emergence and gradually increases with root elongation (Fig. 4), further showing ethylene to be a consequence of the germination process. Additional seed lots and test conditions are needed to unravel the actual function of ethylene during the germination process of sugar beet. In other species (e.g. lettuce) ethylene is proposed to act by promoting radial cell expansion in the embryonic axis or by increasing the water potential or seed respiration (Kucera *et al.*, 2005; Matilla and Matilla-Vázquez, 2008). In *Lepidium sativum* and *Arabidopsis thaliana*, ethylene biosynthesis and signalling play an important role during endosperm cap weakening by counteracting ABA inhibition (Linkies *et al.*, 2009). Linkies and Leubner-Metzger (2012) proposed, therefore, that in future research on ethylene action during seed germination, tissue-specific mechanisms and interactions with other hormones must be considered.

The role of ethylene during sugar beet germination has been investigated previously by Hermann *et al.* (2007). In apparent contrast with our results, they concluded that ethylene promotes sugar beet germina-

Table 1. Effect of aminooxyacetic acid (AOA), 2-amino isobutyric acid (AIB), silver thiosulphate (STS) and 1-aminocyclopropane-1-carboxylic acid (ACC) on the ethylene production of intact sugar beet fruits 72 h after the start of imbibition. Each value represents the mean of five independent repeats of 20 fruits, incubated at 20°C. Mean values ± SD are presented

Treatment	Ethylene production (pl h^{-1} fruit^{-1})
Control	88.3 ± 8.7
1 mM AOA	33.5 ± 2.7
1 mM AIB	71.3 ± 7.2
1 mM STS	136.1 ± 11.3
1 mM ACC	696.5 ± 65.1

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tion, on the basis of the effects of applied ethephon (release of ethylene), ACC and the ethylene signalling inhibitor 2,5-norbornadiene. In addition, they reported an accumulation of ACO-transcripts at t_{50} in fruits and seeds during germination to reinforce their conclusion. In our study, no endogenous ethylene release was detected during the first 48 h after imbibition of intact fruits, in contrast with the ethylene release found afterwards. All this clearly indicates that an increase in ACO-transcripts is not equal to an increase in ethylene production rate. The effects of applied ACC and the ethylene signalling inhibitor STS on the germination pattern in our study are different to those described by Hermann *et al.* (2007). This can be explained by the different genetic background of the analysed sugar beet hybrids (e.g. diploid versus triploid). Genotypic variation on hormonal responses has already been observed in sugar beet callus (Jarl and Bornman, 1986).

In conclusion, our study clearly demonstrates that ethylene release in sugar beet results from germination rather than acting as a prerequisite for the germination process. However, also in sugar beet, hormonal responses can differ strongly between different genotypes.

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