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BRIEF COMMUNICATION

Evaluation of Viability, Shedding Pattern, and Longevity of Pollen from Genetically Modified (GM) Herbicide-tolerant and Wild-type Zoysiagrass (*Zoysia japonica* Steud.)

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Abstract Knowledge about pollen viability is important when evaluating the risk of genetically modified (GM) plants. Here, staining via iodine potassium iodide (IKI) or triphenyl tetrazolium chloride (TTC) could not distinguish between live and dead pollen from *Zoysia japonica*. Therefore, to obtain a reliable assessment of such viability and longevity, we developed an optimum germination medium containing 20% sucrose and 50 ppm H_3BO_3. Pollen grains transferred to the germination medium at about 1000 hours had a germination rate of >90%. Pollen was most predominantly shed at approximately 1000 hours, with viability declining to nearly 0% at 1200 hours. All germinability was lost within 150 min when stored at 25°C. No significant difference was found between GM and non-GM plants in their pollen viability or longevity.

Keywords Gene flow · Genetically modified (GM) · Pollen · *Zoysia japonica*

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Introduction

Turf grass has enormous economic value worldwide, especially in developed countries. Various genera have long been utilized for courtyards, parks, golf courses, and athletic fields. The warm-season Korean grass, *Zoysia japonica*, is one of the most commonly grown, particularly in East Asia, because it is tolerant to drought and disease and makes an ideal lawn for sports and recreational settings (Bae et al. 2008).

Many genetically modified (GM) crops have been generated and commercialized (Halpin 2005; Owen and Zelaya 2005; Christou et al. 2006; Bhatnagar-Mathur et al. 2008). For commercial purposes, these GM crops must be assessed and monitored for their potential environmental risks, including the possibility of pollen-mediated gene flow into wild relatives (Lefol et al. 1996; Lavigne et al. 2002; Snow 2002; Fei and Nelson 2003). Transgenic zoysiagrasses have been produced via GM crop technology (Inokuma et al. 1998; Toyama et al. 2003; Ge et al. 2006; Li et al. 2006). Focused on ecological and environmental concerns, a biosafety assessment of GM herbicide-tolerant zoysiagrass was performed (Bae et al. 2008). There, pollen-mediated gene flow was examined only by evaluating the herbicide tolerance of progeny from potentially pollinated grasses. However, basic characteristics, e.g., pollen viability, longevity, and shedding pattern, have not yet been measured, although they are important in providing guidelines for reducing the potential for pollen-mediated gene flow (Bae et al. 2008). *Z. japonica* tends toward clonal propagation by stolons. It also has a protogynous flower that is wind-pollinated such that seeds are produced after fertilization by neighboring plants rather than through self-pollination. Therefore, zoysiagrass pollen becomes a carrier

Table 1 The effect of sucrose and H_3BO_3 concentrations on pollen germination of zoysiagrass

H_3BO_3(ppm)	Sucrose (%)					
	0	3	9	12	18	24
0	1.3(1.5)[a]	6.9(1.2)	14.4(4.0)	17.0(3.5)	13.5(0.4)	12.5(0.3)
25	2.4(0.2)	1.2(0.5)	8.5(3.0)	10.3(2.6)	25.8(1.5)	24.9(4.8)
50	3.9(3.0)	4.2(2.0)	12.1(2.5)	8.8(2.1)	20.3(7.4)	25.9(2.9)
100	1.7(0.7)	0.8(0.1)	6.3(2.1)	12.3(1.9)	6.3(3.5)	11.1(7.3)
250	1.1(0.6)	1.1(0.1)	16.5(1.9)	19.9(2.5)	11.6(9.9)	12.0(9.5)
500	0.3(0.3)	5.3(6.2)	7.3(3.6)	1.0(0.5)	1.9(1.3)	2.8(0.8)

[a] Values are germination percentage (SE)

that would enable a transgene to deliver into surrounding wild relatives. The longevity of pollen after anther dehiscence is crucial to successful pollination (Stone et al. 1995), particularly for outcrossing preferential zoysiagrass.

Pollen management has been studied as a method to control gene flow in transgenic maize (Luna et al. 2001). Understanding its viability, longevity, and shedding pattern is helpful in developing means for controlling pollen travel (Fei and Nelson 2003). Viability can be estimated by staining it with dyes such as iodine and potassium iodide (IKI) (Song et al. 2001), triphenyl tetrazolium chloride (TTC) (Huang et al. 2004), or fluoresceine diacetate (Eady et al. 1995). However, these do not always apply coincidentally for many plant species (Mulugeta et al. 1994). Therefore, pollen germination on an artificial medium has been established as a more faithful approach for evaluating viability in grass family members, including ryegrass (Ahloowalla 1973), Kentucky bluegrass (Teare et al. 1970), maize (Geetha et al. 2004), and creeping bentgrass (Fei and Nelson 2003). However, this technique has not been tested yet with zoysiagrass.

Here, we developed an artificial medium for estimating the viability and longevity of pollen from herbicide-tolerant GM and non-GM zoysiagrass plants.

Materials and Methods

Plant Materials

Zoysiagrass (*Z. japonica* Steud.) was used in this study (Bae et al. 2001, 2008). Briefly, herbicide-tolerant plants were generated using pGPTV-HB (Bae et al. 2001; Toyama et al. 2003) according to the method developed by Toyama et al. (2003). The transgenic line contained a single-copy T-DNA. Plants were vegetatively propagated and maintained in test fields and confined vinyl houses at Jeju National University as approved for environmental risk assessments of GM organisms by the Rural Development Administration of Ministry for Food, Agriculture, Forestry, and Fisheries (MFAFF) of Korea. Watering was provided from

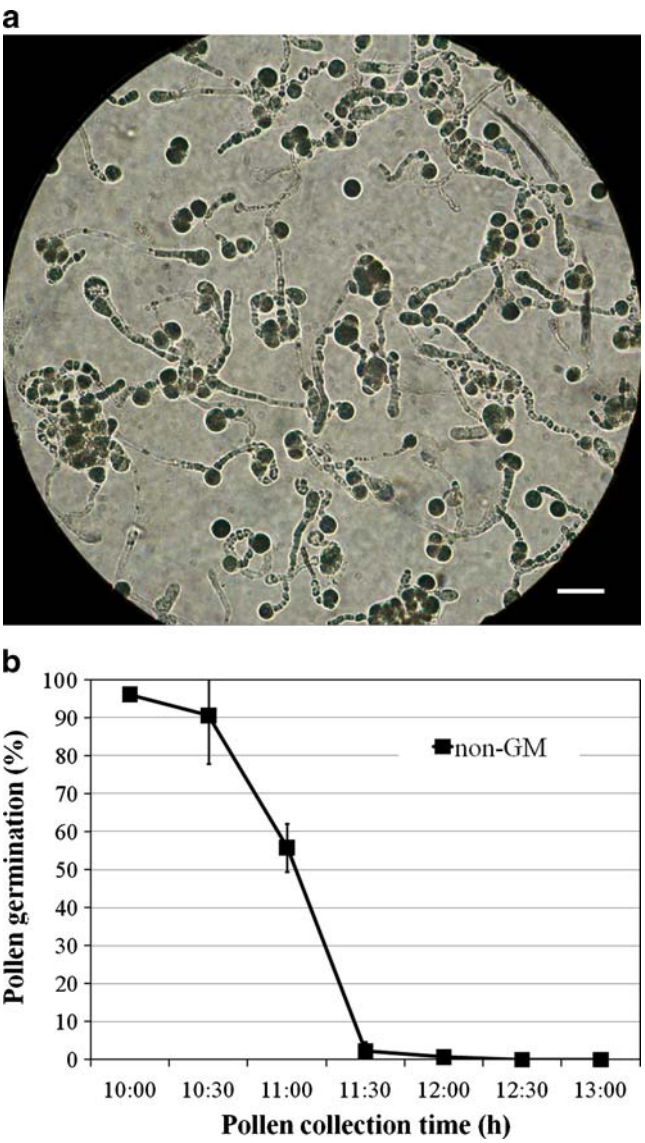

Fig. 1 Shedding pattern based on in vitro tests. **a** Pollen germination from non-GM *Zoysia japonica* on medium containing 20% sucrose and 50 ppm H_3BO_3. Representative microscopic image after 6 h incubation. **b** Germination percentage over 3 h, from 1000 to 1300 hours. Immediately after each collection, pollen grains were transferred to suitable germination medium and incubated at 25°C under fluorescent light. *Bar* 100 μm

a sprinkler to avoid drought stress, and plants were treated periodically with compound fertilizer to prevent nutrient deficiencies. Most experiments were performed during the natural flowering season, late April to mid-May.

Staining Experiments for Pollen Viability

Staining methods that utilized TTC and IKI were checked to determine whether they are effective for viability tests of pollen from *Z. japonica*. Grains were stained for either 2 h in 1% TTC solution (Norton 1966) or for 30 min at room temperature in IKI solution (1 g KI and 0.5 g I_2 dissolved in 100 ml of distilled water; Eti 1991). After transfer to slide glass, they were covered with a slip and observed under a light microscope (Axiostar Plus; Carl Zeiss, Germany).

Germination Experiments for Pollen Viability

To identify the appropriate artificial medium for investigating pollen germination or viability, we performed a factorial experiment involving six sucrose concentrations (0%, 3%, 9%, 12%, 20%, and 24%) and six H_3BO_3 concentrations (0, 25, 50, 100, 250, and 500 ppm), for a total of 36 media. After each medium was supplemented with Phytogel (Sigma, St. Louis, MO) at 3 g L^{-1} and dissolved in a microwave oven, it was dispensed into 35×10 mm Petri dishes. Pollen grains were incubated for approximately 6 h on these media in a 25°C room under fluorescent light until pollen tubes were fully elongated. They were then stored at 4°C. More than 300 grains from three fields per dish were counted under the light microscope.

The pattern of pollen-shedding was monitored between 1000 and 1300 hours, at 10- to 60-min intervals. Longevity of grains collected between 1000 and 1100 hours was examined either under sunny outside conditions or at 25°C. All experiments described here entailed three replications.

Results and Discussion

Assessment of Pollen Viability via Staining

Pollen staining and germination methods were evaluated to determine whether such tests would be helpful for assessing the pollen-mediated gene flow of GM zoysiagrass. Staining with IKI and TTC are commonly used to estimate viability (Mulugeta et al. 1994; Dafni and Firmage 2000). About 90% of the grains from both GM and non-GM zoysiagrass stained purple brown, and this coloring did not disappear. No significant difference was found between the pollen from either genotype. Moreover, even heat-killed grains stained in the same manner as fresh pollen, indicating that IKI staining is not appropriate for effectively distinguishing between viable and dead pollen from this species. However, this method remains suitable for examining grain development because starch granules generally are found only in normal pollen.

TTC staining was also performed, but compared with IKI, staining patterns showed strong fluctuations, resulting in no reproducibility (data not shown). For many species, this TTC method is helpful for estimating pollen viability (Mulugeta et al. 1994; Huang et al. 2004), whereas for others, it can yield false-positive scores for viability when compared with data from germination tests (Stone et al. 1995; Wang et al. 2004; Dafni et al. 2005). Here, neither staining protocol proved appropriate for determining the pollen viability of *Z. japonica*.

In Vitro Germination of Pollen from *Z. japonica*

For a more reliable estimate of viability, we performed in vitro germination tests on an artificial medium. Generally, such experiments are associated with treatments that use sucrose and H_3BO_3 and $CaCl_2$ or $Ca(NO_3)_2$ (Wang et al.

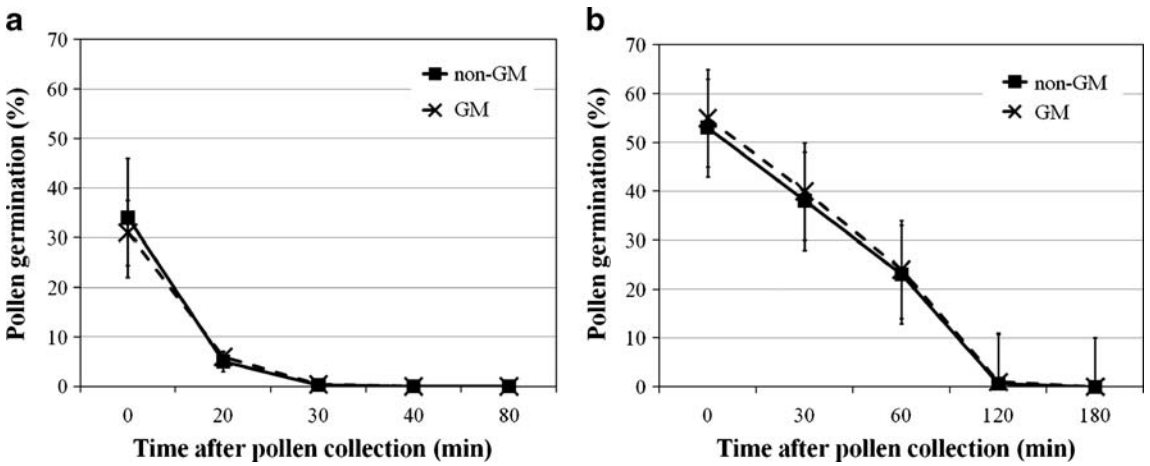

Fig. 2 Longevity estimates of pollen grains from GM herbicide-tolerant and non-GM plants based on in vitro tests. **a**, **b** Experiments under sunny outside and controlled (25°C) conditions, respectively. *Vertical bars* show standard error

2004). However, in our preliminary trial, sucrose and H_3BO_3 had an evident effect on the rate of pollen germination, while $Ca(NO_3)_2$ did not. We assessed 36 media combinations—six sucrose concentrations (0%, 3%, 9%, 12%, 18%, or 24%) plus six H_3BO_3 concentrations (0, 25, 50, 100, 250, or 500 ppm). Without sucrose, germination was very difficult, but the rate increased with higher sucrose concentrations (Table 1). The near-best levels of sucrose and H_3BO_3 were 18–24% and 25–50 ppm, respectively. Therefore, based on this factorial experiment (see Fig. 1a), we selected a medium containing 20% sucrose and 50 ppm H_3BO_3 as most suitable for further evaluations. Almost all of pollen grains or tubes were intact, not ruptured after 6 h incubation on the suitable medium.

Estimation of Pollen Shedding and Longevity by In Vitro Germination

Pollen germination on an artificial medium is the most valid means for determining in vivo viability (Stone et al. 1995). Here, grains transferred to our suitable germination medium at about 1000 hours had a germination rate of >90% (Fig. 1b). This is believed to be the time of day when anthers dehisce. Afterward, the germination rate decreased rapidly, to about 55% at 1130 hours and to <5% at 1200 hours (Fig. 1b). No germination occurred from 1230 to 1300 hours (Fig. 1b). Creeping bentgrass (*Agrostis*), a turfgrass grown mainly on golf course greens, has two peaks of pollen viability—0900 and 1400 hours (Fei and Nelson 2003). Here, however, we did not identify a daily shedding pattern for *Z. japonica* because pollen viability was checked only until 1300 hours. Nevertheless, the shedding pattern for zoysiagrass differed from that of creeping bentgrass.

The longevity of pollen was compared between GM herbicide-tolerant zoysiagrass and non-GM plants under natural conditions (Fig. 2a) and at 25°C (Fig. 2b). Differences were not significant in this evaluation (Fig. 2a, b). Initial germination rates were about 35% (natural) and 55% (controlled environment). Pollen grains collected between 1000 and 1100 hours were transferred to our suitable germination medium at 10- to 60-min intervals. This delay between pollen collection and start of analysis meant that the germination rate at each time point was not constant (Fig. 2a, b). For example, under natural conditions, the rate dropped to 0% at approximately 40 min from the beginning of the experiment, whereas the rate at 25°C was about 25% at 60 min and did not decrease to 0% until 180 min. This indicated that viability was lost more rapidly in the natural environment. Such short longevity has also been reported with bentgrass (Fei and Nelson 2003) as well as *Zea mays* L., which loses viability after 2 h under field conditions (Luna et al. 2001). In addition, no seed is set after 5 h for *Sorghum bicolor* L. (Stephens and Quinby 1934).

In summary, we have now developed a simple and effective pollen germination medium for evaluating pollen from *Z. japonica*. Here, the maximum germination rate was >90%. No significant difference was found between GM herbicide-tolerant and non-GM zoysiagrasses in their pollen viability or longevity. This knowledge about pollen shedding and longevity will provide valuable information for breeding and assessing the risk of genetically modified crops for this species.

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