

## **Fruit Maturity Effects and Dormancy Breaking Treatments in High lycopene and Normal Tomato Seed**

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### **Abstract**

The tomato fruit is an important source of carotenoids such as  $\beta$ -carotene and lycopene. Carotenoids play important roles in nutrition and human health, such as evidenced by high correlation between lycopene content and reduced risk of prostate cancer. High pigment genes such as *dark green* (dg) and *high pigment* (hp) that result in higher levels of lycopene in tomato fruit are available. However, their deleterious effects on plant development (such as reduced plant height and slow germination) have limited their use as homozygotes in commercial tomato varieties. The cause(s) of these effects is not well understood. This study evaluated fruit development effects on seed quality of a dg line, its recurrent parent and a wild type. Tomato plants were grown under greenhouse conditions for fruit and seed production. Fruits were harvested at five maturity stages (Mature Green, Breaker, Pink Breaker, Red Mature and Overripe). Seed quality was evaluated using germination index, standard germination and saturated salt accelerated aging tests. In another experiment, gibberellin and norflurazon (an inhibitor of carotenoid synthesis) and light effects on time to 50% germination and germination index were evaluated. Results indicated that the low speed of germination of the dg line is independent of the gradual accumulation of lycopene. Seeds of the dg line treated with norflurazon plus gibberellin germinated faster than the control, suggesting that ABA is involved in the low speed of germination of dg genotypes. Germination of the dg genotype was higher under darkness and values were similar to those obtained using the ABA inhibitor. These findings suggest that germination of dg tomato genotypes is mediated by light, possibly via regulation of ABA synthesis after imbibition.

### **Introduction**

Tomato (*Lycopersicon esculentum* Mill.) fruits are important sources of antioxidants, such as lycopene that prevent various types of cancers (Van Der Berg *et al.*, 2000). Because antioxidants function as free radical scavengers, tomato breeders are now developing plant materials with high lycopene content. However, increasing carotenoid and lycopene levels using traditional breeding and molecular biology approaches might also alter other metabolic pathways and cause undesirable abnormalities in normal plant development (Croteau *et al.*, 2000). For example,

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delayed germination and reduced plant growth have been reported in the tomato mutants high pigment (hp) and dark green (dg) mutants in which lycopene content is much higher (2-3X) than normal tomato genotypes (Jarret *et al.*, 1984; Wann *et al.*, 1985; Berry and Uddin, 1991). In addition, high levels of carotenoids (a group of naturally occurring antioxidant) might decrease the synthesis of essential germination promoters such as gibberellins (Fray *et al.*, 1995; Croteau *et al.*, 2001) leading to a reduced speed of germination in high pigment tomato genotypes. Because lycopene synthesis increases as the fruit matures (Giovannelli *et al.*, 1999; Arias *et al.*, 2000), elevated levels of lycopene may be responsible for the reduced speed of germination of the dg and hp mutants. As a result, additional research is still required to determine whether a direct relationship exists between increased lycopene synthesis during fruit development and maturity and seed germination and longevity.

The reduced plant growth of dg and hp genotypes has been associated with low endogenous gibberellin content (Wann, 1995). Gibberellins are required for synthesis of hydrolytic enzymes and endosperm weakening, both processes are required for radicle protrusion in tomato seeds (Bradford *et al.*, 2000). Unfortunately, there is no direct evidence that the low speed of germination of high lycopene tomato mutants is caused by low endogenous gibberellin content. The study by Wann (1995) evaluated the effect of gibberellin in plant height of dg and hp mutants but did not consider its effect on seed germination. Since carotenoids are precursors of ABA via an indirect pathway (Zeevaart, 2000), it is possible that this delay in seed germination is caused by higher levels of abscisic acid (ABA). The objectives of this research were to (i) evaluate fruit development effects on tomato seed quality of a normal and dg tomato genotype and (ii) evaluate the effects of gibberellin and an inhibitor of ABA on speed of germination of dg tomato genotypes.

## **Materials and Methods**

### *Fruit Development Effects on Seed Quality.*

Seeds of two unrelated tomato genotypes differing in seed vigor and lycopene synthesis and accumulation were planted in a greenhouse in winter 2000 and summer 2001. The winter study included the open pollinated variety 'OH8245' developed at The Ohio State University and the high lycopene line 'T4099' (dg) developed at The United States Department of Agriculture (USDA) by backcrossing with the open pollinated variety 'Flora-Dade' followed by several generations of self-pollination (Wann, 1996). Fruits were harvested at five maturity stages (mature green, breaker, pink-breaker, red fruit and overripe) following the criteria described by Valdes and Gray (1998). Seeds were extracted by hand and fermented in a beaker at room temperature (~24 °C) for 48 h.

The summer 2001 study included the recurrent parent 'Flora-Dade' and the experimental line 'T4099' dg/og<sup>c</sup>. Fruits were harvested and seeds extracted as described above. For the winter 2000 study, seed quality was evaluated by standard germination, germination index (GI) and saturated salt accelerated aging (SSAA) tests (Jianhua and McDonald, 1996). The GI was calculated for each treatment by the algebraic sum of the ratio of normal germinated seedlings and the day after planting at which time the count was made. The standard germination and the GI were evaluated up to 14 days (ISTA, 1999). Fifty seeds were planted in each of four Petri plates

containing two layers of blotter paper saturated with 10 ml demineralized distilled water. The plates were then placed in a germination chamber at 25 °C with a 16/8 h light and dark cycle. In all cases, seeds were recorded as germinated when they produced the essential structures to be considered normal seedlings. For SSAA, seeds were aged in a chamber at 41 °C and 75 RH for 96 h. After this treatment, seeds were evaluated for standard germination.

For the summer 2001 study, seed quality was evaluated by the standard germination test, which was conducted on dry and fresh seeds (seeds extracted after the fermentation process without desiccation). Germination conditions were as described previously. Percentage of germinated seeds (radicle protrusion) and percentage of normal seedlings were determined after 5 and 14 d, respectively.

The experimental design was a complete randomized design with four (winter 2000) and two (summer 2001) replications as repeated measures (Hinkelmann and Kempthorne, 1994).

*Gibberellin, Norflurazon and Light Effects.*

Four replications (50 seeds each) were planted in Petri dishes containing either a solution of dd water (control),  $10^{-4}$  M gibberellin (GA<sub>3</sub>) (Aldrich Milwaukee WI), 20 mg/L of norflurazon (Supelco, Bellefonte, PA) or gibberellin plus norflurazon. Petri dishes were placed in a germination chamber at 24 °C and 16/8 light/darkness cycle.

Treatments effects on germination were measured as the germination index, hypocotyl length and time to 50% germination (T50). Germination index was calculated as the algebraic sum of the ratio obtained dividing the number of seeds showing radicle protrusion and the days after sowing. Hypocotyl length was measured in cm 8 days after sowing using 10 seedlings per replication. Time to 50% of visible germination (T50) was calculated using probit analysis on time (SAS Institute, 2001).

Data were analyzed as a completely randomized design in a factorial arrangement with four replications.

For light and darkness studies, two experiments were conducted. In experiment 1, seeds of 'Flora-Dade' and 'T4099' were germinated in Petri dishes filled with 10 ml demineralized distilled water and covered with aluminum foil for 24, 48, 72, 96, and 120 h. Three replications of 50 seeds each were sown for each period and visible germination (radicle protrusion) was recorded at the end of each period. The control consisted of germination under 16/8 h dark/light cycles. The effect of treatments on germination was measured as the germination index, and time for 50% germination (T50). In experiment 2, seeds of both genotypes were germinated in darkness as in experiment 1, but the light treatment consisted of germination in 16/8 h light/night cycles.

The experimental design was a completely randomized design with factorial arrangement. Data were analyzed using proc GLM and Least Squares Means. Main effects were genotypes ('Flora-Dade' and 'T4099'), light treatment (light and darkness), and experiments (16/8 h darkness/light experiment 1, 16/8 h light/darkness experiment 2).

## **Results**

The experimental line 'T4099' had a lower germination index at all fruit maturities ( $p < 0.01$ ) than 'OH8245' (Table 1) indicating lower speed of germination. However, 'T4099' had a higher final germination percentage than 'OH8245' at the mature green and breaker stages (Table 2). Genotype 'OH8245' showed higher germination at the red mature and the overripe stages ( $p < 0.01$ ) indicating differences in the effect of fruit maturity on seed quality between these two genotypes. Germination percentage of 'T4099' increased from mature green to breaker and then declined resulting in 85.2% germination at the overripe stage. On the other hand, germination of 'OH8245' increased from mature green to red mature fruit stages and then declined. The SSSA test showed that 'T4099' was more susceptible to artificial deterioration, especially in the overripe stage (Table 3). The overripe stage resulted in 62% germination after SSAA compared to 95% germination for 'OH8245'. This genotype followed the general pattern that red mature fruits result in maximum tomato seed quality and after that seeds start to deteriorate (Valdes and Gray, 1998; Demir and Samit, 2001). Apparently, 'T4099' followed a different pattern with the mature green and breaker stages having higher seed quality.

Results of the summer study indicated that after 5 d of imbibition, 'Flora-Dade' had a higher germination percentage (radicle protrusion) for fresh seeds than 'T4099'. However, only at the overripe stage was this difference significant (Table 4). Similarly, 'Flora-Dade' showed a higher percentage of normal seedlings than 'T4099' at all fruit maturities (Table 5). Differences between 'Flora-Dade' and 'T4099' were more marked for seedling growth than for radicle protrusion. In 'Flora-Dade', percentage germination increased from mature green to red mature fruit stages and remained constant from red mature to overripe fruit maturity. Germination was greater than 90% in red mature and overripe stages. In 'T4099', germination increased from mature green to pink breaker; however, germination was never greater than 25%. This difference at 5 d between 'Flora-Dade' and 'T4099', which is more obvious for normal seedlings, was consistent with the low germination index found in the winter study and demonstrates the inherently low seed vigor of genotype 'T4099'. For dry seeds, 'Flora-Dade' showed a higher percentage germination (radicle protrusion) than 'T4099' in all fruit maturities except the overripe stage (data not shown). Similarly, for fresh seeds, the difference in percentage germination was greater for normal seedlings compared to radicle protrusion.

### *Gibberellin, Norflurazon and Light Effects.*

Genotype 'Flora-Dade' initiated germination 2 days earlier than 'T4099' (Table 6). 'Flora-Dade' had 60-75% germination two days after sowing. In contrast, 'T4099' showed less than 10% at the same time after sowing (Table 6). By 3 DAS, seeds treated with gibberellin plus norflurazon had approximately 35 % greater germination than the control while norflurazon had only 20 % germination. The speed of germination measured by T50 and germination index indicated that the best treatments were gibberellin plus norflurazon (Table 7). None of the treatments (norflurazon, gibberellin, or norflurazon plus gibberellin) improved germination percentage of 'T4099' to the same level as 'Flora-Dade'. However, there was a positive response of 'T4099' to gibberellin and norflurazon, especially when they were applied simultaneously.

'Flora-Dade' had higher germination than 'T4099' during the first 2 to 3 days regardless of light treatment. However, in both genotypes, germination under darkness was higher than under light. Germination under 16/8 h dark/light cycles resulted in higher germination than 16/8 h light/dark cycles, and this effect was more marked for 'T4099' (Figure 1). For 'Flora-Dade', 16/8 h light/dark cycles resulted in a T50 value of 1.95 versus 1.58 for darkness and lowest germination index 61.5 versus 70.3. For 'T4099', 16/8 h light/dark cycle resulted in a T50 of 3.82 versus 2.97, and a germination index of 28.4 versus 40.3 for darkness (Table 8). The difference for 'T4099' between darkness and 16/8 h dark/light cycle was 0.32 days and that for 'Flora-Dade' was 0.20 days (Table 8). In addition, the difference between darkness and 16/8 h light/dark cycle was 0.85 days for 'T4099' and 0.37 for 'Flora-Dade'.

## **Discussion**

Because tomato fruit maturity is accompanied by an increase in lycopene synthesis and accumulation, it was necessary to determine whether harvesting 'T4099' in the early stages of fruit maturity (i.e. breaker stage) would improve the speed of germination. Germination index values indicated that 'T4099' had lower speed of germination compared to 'OH8245' regardless of fruit maturity (Table 1). This finding indicates that the speed of germination of this line is not improved by harvesting at the early stages of fruit maturity and suggests that the effect of the dg gene in germination physiology is independent of the gradual accumulation of lycopene that accompanies fruit development. Genotypes contrasting in lycopene synthesis and production were included to evaluate whether a difference in fruit development on seed quality in a traditional variety and a high lycopene variety was present. Germination of 'T4099' was less affected by the mature green fruit stage than for 'OH8245'. In addition, red mature and overripe fruit stages resulted in lower seed quality in 'T4099' compared to 'OH8245' (Table 2). This finding suggests that 'T4099' acquires maximum germination capacity in early fruit maturity stages and that red mature and overripe fruit maturities might have a detrimental effect on the final germination percentage of 'T4099'.

The differences between 'Flora-Dade' and 'T4099' was more marked for seedling growth than for radicle protrusion, suggesting the dg gene is more critical for seedling development than for germination. This difference at 5 d between 'Flora-Dade' and 'T4099', which is more obvious for normal seedlings, was consistent with the low germination index found in the winter study and demonstrates the lower seed vigor of 'T4099'. The effect of the dg gene is observed in slower radicle protrusion and reduced seedling and plant growth, resulting in a shorter plant compared to the wild type. This finding suggests that delayed radicle protrusion, slow seedling development and reduced plant growth are effects caused by a common factor in dg genotypes. Given that gibberellins play an important role on germination, cell elongation and plant growth, they are perhaps the best candidates. However, germination also involves ABA; therefore, the possibility exists that ABA is also involved in the low speed of germination of dg genotypes.

Because norflurazon is an inhibitor of carotenoid synthesis (Breitenbach *et al.*, 2001), and consequently ABA, the different norflurazon effect between 'Flora-Dade' and 'T4099' suggests that ABA produced after imbibition is possibly greater in 'T4099' than in 'Flora-Dade' which results in delayed seed germination. The difference in ABA levels is possibly due to the greater carotenoid content in seeds of 'T4099' compared to those of 'Flora-Dade'. The fact that

norflurazon, or gibberellin plus norflurazon did not improve speed of germination of 'T4099' to the same level as 'Flora-Dade' seeds suggests that another mechanism is involved, possibly ABA produced during seed development (Debeaujon and Koorneef, 2000).

For 'Flora-Dade', 16/8 h light/dark cycles resulted in a T50 value of 1.95 versus 1.58 for darkness and lowest germination index values (61.5 versus 70.3). For 'T4099', 16/8 h light/dark cycles resulted in a T50 of 3.82 versus 2.97, and a germination index of 28.4 versus 40.3 for darkness (Table 8). These results clearly suggest that the germination of a dg genotype is improved by darkness and that this effect is higher in 'T4099'. These results also suggest that 'T4099' shows a higher light sensitivity than 'Flora-Dade' and this hypersensitivity is probably mediated by phytochrome A as previously observed for the tomato hp mutant (Shichijo *et al.*, 2001). Interestingly, gibberellin plus norflurazon resulted in similar values for T50 and germination index (2.86 and 41.8, respectively) to those for darkness (2.97 and 40.3, respectively) (Tables 7 and 8). This finding indicates that darkness, and gibberellin plus norflurazon may improve the speed of germination of 'T4099' by a common mechanism, such as the regulation of ABA biosynthesis. Regulation of ABA biosynthesis by light may be related to the activity of the enzymes 9 cis-epoxycarotenoid dioxygenase (NCED) and zeaxanthin epoxidase (ZEP) mRNAs (Thomson *et al.*, 2000). Tomato genotypes that produce fruits with elevated levels of lycopene might result in a delayed seed germination caused by higher level of ABA than normal tomatoes.

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Table 1. Germination index of ‘OH8245’ and ‘T4099’ tomato seeds harvested at five different fruit maturities: mature green (MG), breaker (BR), pink breaker (PB) red mature (RM) and overripe (OR). Winter 2000.

<b>Genotype</b>	<b>MG</b>	<b>BR</b>	<b>PB</b>	<b>RM</b>	<b>OR</b>
‘OH8245’	15.8 a	18.4 a	18.2 a	19.5 a	18.9 a
‘T4099’	12.2 a	13.1 a	12.5 a	12.2 b	11.0 b

<sup>Z</sup> Values within columns with the same letter are not significantly different at  $\alpha$  0.05.

Table 2. Germination percentage of ‘OH8245’ and ‘T4099’ tomato seeds harvested at five different fruit maturities: mature green (MG), breaker (BR), pink breaker (PB) red mature (RM) and overripe (OR). Winter 2000.

<b>Genotype</b>	<b>MG</b>	<b>BR</b>	<b>PB</b>	<b>RM</b>	<b>OR</b>
‘OH8245’	86.7 b	90.7 b	94.7 a	99.2 a	96.2 a
‘T4099’	92.5 a	95.7 a	91.5 a	90.5 b	85.2 b

<sup>Z</sup> Values within columns with the same letter are not significantly different at  $\alpha$  0.05.

Table 3. Germination percentage after SSAA of ‘OH8245’ and ‘T4099’ tomato seeds harvested at five different five fruit maturities: mature green (MG), breaker (BR), pink breaker (PB) red mature (RM) and overripe (OR). Winter 2000.

<b>Genotype</b>	<b>MG</b>	<b>BR</b>	<b>PB</b>	<b>RM</b>	<b>OR</b>
‘OH8245’	80.2 a	84.5 a	95.0 a	97.5 a	95.0 a
‘T4099’	83.5 a	82.5 a	82.5 a	75.5 b	63.0 b

<sup>Z</sup> Values within columns with the same letter are not significantly different at  $\alpha$  0.05.

Table 4. Five-day count percentage germination (radicle protrusion) of fresh tomato seeds of ‘Flora-Dade’ and ‘T4099’ harvested at five fruit maturities: mature green (MG), breaker (BR), pink breaker (PB) red mature (RM) and overripe (OR)). Summer 2001. <sup>Z</sup> values within columns with the same letter are not significantly different at  $\alpha$  0.05.

<b>Genotypes</b>	<b>MG</b>	<b>BR</b>	<b>PB</b>	<b>RM</b>	<b>OR</b>
‘Flora-Dade’	92.7 a <sup>Z</sup>	98.0 a	90.5 a	99.0 a	95.0 a
‘T4099’	73.2 a	93.0 a	76.0 a	97.0 a	59.0 b

<sup>Z</sup> Values within columns with the same letter are not significantly different at  $\alpha$  0.05.

Table 5. Five-day count percentage germination (normal seedlings) of fresh tomato seeds of ‘Flora-Dade’ and ‘T4099’ harvested at five fruit maturities: mature green (MG), breaker (BR), pink breaker (PB) red mature (RM) and overripe (OR)). Summer 2001.

<b>Genotype</b>	<b>MG</b>	<b>BR</b>	<b>PB</b>	<b>RM</b>	<b>OR</b>
‘Flora-Dade’	15 a	35.5 a <sup>z</sup>	75.5 a	92.0 a	92.7 a
‘T4099’	0 b	3.75 b	21.2 b	10.0 b	17.7 b

<sup>z</sup>Values within columns with the same letter are not significantly different at  $\alpha$  0.05.

Table 6. Percentage germination (radicle protrusion) of seeds from two tomato genotypes (‘Flora-Dade’ and ‘T4099’) treated with solutions of gibberellin, norflurazon, and gibberellin plus norflurazon.

<b>Genotype/Treatment</b>	<b>Days</b>				
	1	2	3	4	5
‘Flora-Dade’ / Control	0	66	97	100	100
‘Flora-Dade’ / GA <sub>3</sub>	0	58	96	100	100
‘Flora-Dade’ / Norflurazon	0	72	96	100	100
‘Flora-Dade’ / GA <sub>3</sub> + Norflurazon	0	63	95	100	100
‘T4099’ / Control	0	1	38	87	94
‘T4099’ / GA <sub>3</sub>	0	0	41	92	98
‘T4099’ / Norflurazon	0	1	56	92	95
‘T4099’ / GA <sub>3</sub> + Norflurazon	0	3.5	68	95	97

Table 7. Time to fifty percent germination (T50), germination index (GI) and hypocotyl length (HL) of two tomato genotypes ('Flora-Dade' and 'T4099') treated with solutions of gibberellin (GA<sub>3</sub>), norflurazon (Nor), and gibberellin plus norflurazon.

<b>Treatment</b>	<b>T50 (Days)</b>		<b>GI</b>		<b>HL (cm)</b>	
'Flora-Dade' / GA <sub>3</sub> + Nor	1.96	a <sup>Z</sup>	65.0	a	3.6	b
'Flora-Dade' / GA <sub>3</sub>	1.95	a	61.5	a	4.7	a
'Flora-Dade' / Nor	1.93	a	62.4	a	3.3	c
'Flora-Dade' / Control	1.87	a	64.0	a	3.7	b
'T4099' / GA <sub>3</sub> + Nor	2.86	b	41.8	b	3.9	b
'T4099' / GA <sub>3</sub>	3.18	d	36.4	cd	3.9	b
'T4099' / Nor	2.99	c	38.4	c	2.6	d
'T4099' / control	3.32	e	34.8	d	3.2	c

<sup>Z</sup> Means within column with the same letter are not significantly different at  $\alpha$  0.05.

Table 8. Time to fifty percent germination (T50), and germination index (GI) of seeds from two tomato genotypes: 'Flora-Dade' and 'T4099'. Seeds were germinated under darkness or under 16/8 h dark/light cycles (Experiment 1) and 16/8 h light/dark cycles (Experiment 2). Germination was recorded daily when seeds showed radicle protrusion.

<b>Genotype</b>	<b>Treatment</b>	<b>T50</b>		<b>GI</b>	
'Flora-Dade'	16/8 D/L	1.78	b <sup>z</sup>	66.1	b
'Flora-Dade'	16/8 L/D	1.95	c	61.5	c
'Flora-Dade'	Darkness	1.58	a	70.3	a
'T4099'	16/8 D/L	3.29	e	36.0	e
'T4099'	16/8 L/D	3.82	f	28.4	f
'T4099'	Darkness	2.97	d	40.3	d

<sup>z</sup> Means with the same letter are not significantly different at  $\alpha$  0.05

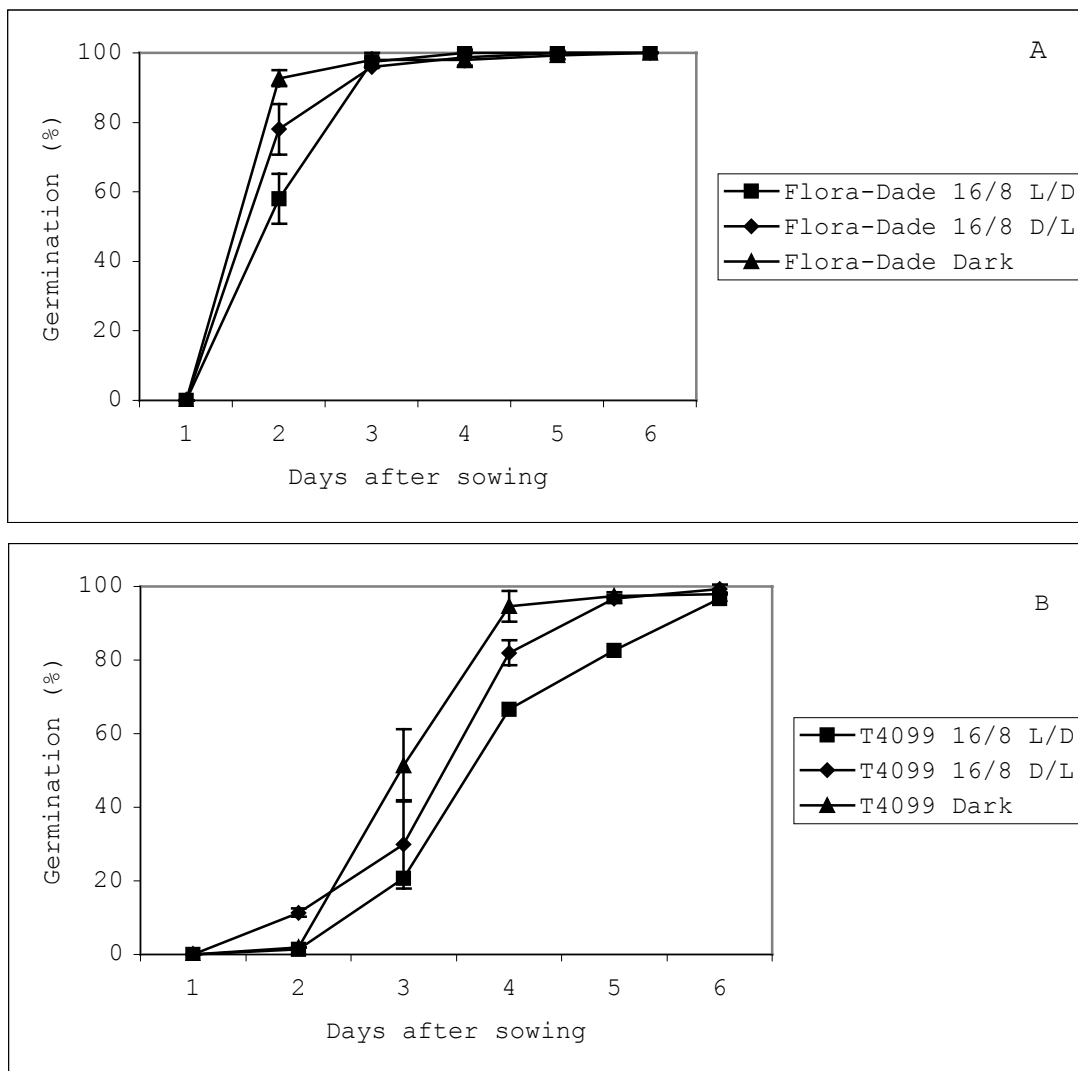


Figure 1. Percentage germination (radicle protrusion) of seeds from two tomato genotypes 'Flora-Dade' (A) and 'T4099' (B). Seeds were germinated under darkness or under 16/8 h dark/light cycles and 16/8 h light/dark cycles.