

# Density-Induced Plant Size Reduction and Size Inequalities in Ethylene-Sensing and Ethylene-Insensitive Tobacco

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**Abstract:** Plant competition for light is a commonly occurring phenomenon in natural and agricultural vegetations. It is typically size-asymmetric, meaning that slightly larger individuals receive a disproportionate share of the light, leaving a limited amount of light for the initially smaller individuals. As a result, size inequalities of such stands increase with competition intensity. A plant's ability to respond morphologically to the presence of neighbour plants with enhanced shoot elongation, the so-called shade avoidance response, acts against the development of size inequalities. This has been shown experimentally with transgenic plants that cannot sense neighbours and, therefore, show no shade avoidance responses. Stands of such transgenic plants showed a much stronger development of size inequalities at high plant densities than did wild type (WT) stands. However, the transgenic plants used in these experiments displayed severely hampered growth rates and virtually no response to neighbours. In order to more precisely study the impact of this phenotypic plasticity on size inequality development, experiments required plants that have normal growth rates and reduced, but not absent, shade avoidance responses. We made use of an ethylene-insensitive, transgenic tobacco genotype (Tetr) that has wild type growth rates and moderately reduced shade avoidance responses to neighbours. Here, we show that the development of size inequalities in monocultures of these plants is not affected unambiguously different from wild type monocultures. Plots of Tetr plants developed higher inequalities for stem length than did WT, but monocultures of the two genotypes had identical CV (Coefficient of Variance) values for shoot biomass that increased with plant density. Therefore, even though reduced shade avoidance capacities led to the expected higher size inequalities for stem length, this does not necessarily lead to increased size inequalities for shoot biomass.

**Key words:** Competition, growth, ethylene perception, plasticity, shade avoidance.

## Introduction

Competition is of major importance in determining the structure and composition of plant canopies and is predominantly by light in dense vegetation. Competition for light is typically asymmetric (Schwinning and Weiner, 1998; Weiner, 1985), meaning that initially larger individuals (e.g., due to faster germination, Black and Wilkinson, 1963) obtain a disproportionate share of the light because they overtop smaller neighbours. This results in reduced light levels for smaller plants, but hardly any light reduction for larger individuals (e.g., Schwinning and Weiner, 1998; Weiner, 1990), thereby even further suppressing the smaller plants. The resulting increased size inequality typically increases with plant density and, thus, with the intensity of competition for light (Ballaré et al., 1994; Weiner, 1985; Weiner et al., 2001). At the same time, plants show plastic growth responses to high plant densities that counteract the development of size inequalities. These allometric shifts include increased relative investment in length growth (Weiner and Thomas, 1992) and are most strongly displayed by the smallest individuals in a population (Weiner and Fishman, 1994; Weiner and Thomas, 1992). The so-called shade avoidance responses (Ballaré, 1999; Smith and Whitelam, 1997) facilitate growth at high plant densities because they lead to a higher position of the leaves in the canopy, which enhances the amount of captured light and decreases shading and suppression by neighbours (Dudley and Schmitt, 1996; Schmitt et al., 1995; Schmitt and Wulff, 1993).

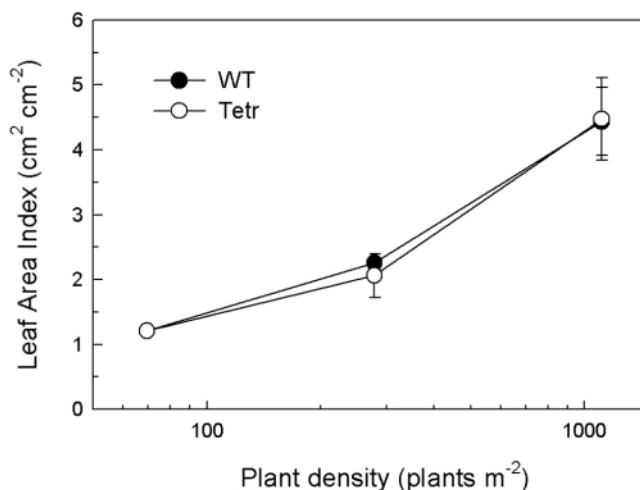
Since shade avoidance responses counteract the development of size inequalities, any attenuation of this shift in allometry towards elongation may lead to increased size inequalities in populations that compete for light. To our knowledge, this has only been shown experimentally by Ballaré et al. (1994) for tobacco. This group found increased size inequalities for shoot biomass and stem length in crowded monocultures of transgenic tobacco plants with inhibited shade avoidance responses. However, they used a genotype that over-expresses oat phytochrome A (PHYA), leading to reduced growth rates and virtually no response to neighbours. A less extreme phenotype would be preferable in such experiments because it would allow us to assess the importance of a more subtle reduction in shade avoidance properties without potential interference with overall growth capacity. We therefore investigated the development of size inequalities in crowded monocultures of transgenic tobacco plants (Tetr) that are insensitive to

the plant hormone ethylene and, as a consequence, have moderately attenuated shade avoidance responses (Pierik et al., 2003). This reveals itself at high densities through an approx. 40% reduced stem elongation rate and a one-week delay in the onset of the hyponastic response (i.e., upward bending of the leaves) (Pierik et al., 2003). Ethylene-insensitive plants typically do not have a dramatically aberrant phenotype under non-stressed conditions because ethylene is often not a prerequisite, but merely a modulator of growth and developmental processes (e.g., Grbic and Bleecker, 1995; Pierik et al., 2003), resulting in wild type growth rates of the Tetr plants (Tholen et al., 2004). Using the Tetr genotype, we tested if attenuated, but not absent, shade avoidance responses to neighbours have consequences for dominance and suppression in dense stands.

## Materials and Methods

Competition experiments were carried out with wild type (WT) and ethylene insensitive, transgenic (Tetr) tobacco. The ethylene insensitive genotype was obtained by Knoester et al. (1998), through introduction of the mutant *etr1-1* ethylene receptor gene from *Arabidopsis thaliana* into tobacco (*Nicotiana tabacum* cv. Samsun NN). The Tetr plants obtained have normal wild type growth rates, but show a delay in the leaf angle and stem elongation responses to neighbours (Pierik et al., 2003). At a high plant density (the highest to be used in the present paper), the onset of the hyponastic response (upward bending of the leaves) to neighbours suffers a delay of approximately one week in Tetr compared to WT, whereas stem elongation rates of Tetr plants (once stem elongation has commenced) are reduced by 40% compared to WT (Pierik et al., 2003).

Seeds were germinated on moist filter paper in petri dishes in a climate room (16 h light,  $240 \mu\text{mol m}^{-2} \text{s}^{-1}$ , Philips PL 600 W), 8 h dark;  $20^\circ\text{C}$ ). After one week, seedlings were selected for homogeneity and transplanted to monocultures of each genotype in even-spaced rectangular plots of  $9 \times 9$  plants at 69, 277 and  $1111 \text{ plants m}^{-2}$ , yielding plot sizes of 1.16, 0.29 and  $0.07 \text{ m}^2$ , respectively. There was no density-induced mortality in any of the treatments. Each combination of density and genotype (six in total) was replicated five times. Each replication of all six treatments was, for practical reasons, transplanted and harvested on a separate day and placed in a separate block. A block consisted of a  $2 \times 2.5 \times 0.25 \text{ m}$  rectangular frame, filled with white river sand and placed in a greenhouse with supplemental lighting (16 h light, minimum light intensity  $220 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 8 h dark, min. temp  $22^\circ\text{C}$ ). Each plot within a block was physically separated below ground from the other plots in the same block. Seedlings that failed to establish were replaced during the first 2 weeks by equal-age plants that had been growing just outside each plot. Plants were watered daily with full strength Hoagland's nutrient solution, to prevent nutrient limitation. The central 9 plants of each plot were harvested 8 weeks after sowing. This was just before the onset of flowering in the highest density plot. Plants in the outer three rows of each plot were not harvested but served to minimise edge effects. At harvest, leaf area and stem length were measured. Shoot dry weight was determined after drying for at least 48 h at  $75^\circ\text{C}$ . The leaf area index (LAI) was determined by dividing the summed leaf area of the 9 plants harvested per plot by the area of soil occupied by these plants. The coefficient of



**Fig. 1** Leaf area index (LAI;  $\text{cm}^2$  leaf area  $\text{cm}^{-2}$  soil surface area) for WT (solid circles) and Tetr (open circles) tobacco monocultures grown at 69, 277 and  $1111 \text{ plants m}^{-2}$  for 8 wks. Data are means ( $n = 5$  plots per genotype per density)  $\pm$  SE.

variation ( $\text{CV} = \text{SD}/\text{mean}$ ) was calculated for biomass and stem length for each replicate plot.

Results of the competition experiments were analysed by means of two-way analysis of variance (SPSS software, version 10.7) with genotype and plant density as fixed variables, with interactions tested, and block as co-variable.

## Results

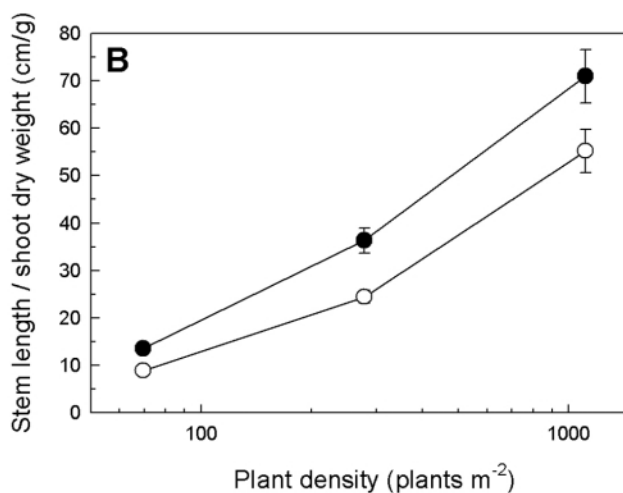
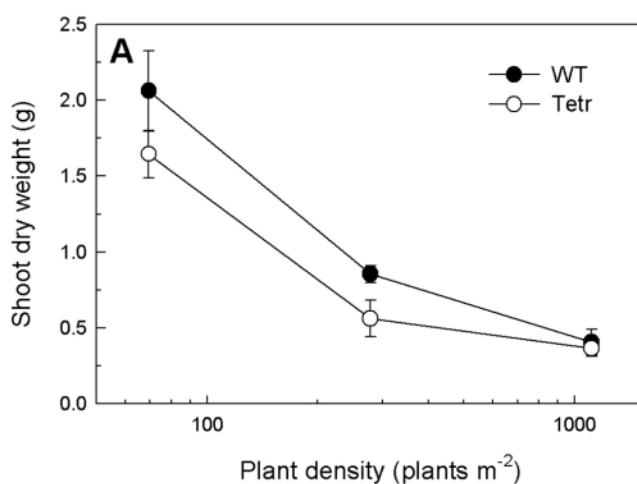
The leaf area index (Fig. 1) increased with increasing plant density from approximately 1 to higher than 4 at the highest plant density. There were no differences in LAI between WT and Tetr canopies (Table 1), indicating that the intensity of aboveground competition was identical in WT and Tetr canopies. Shoot biomass accumulation was severely reduced in both genotypes by increasing plant density (Fig. 2A, Table 1).

Because smaller shoots (caused by reduced biomass accumulation at increasing plant density) tended to have shorter stems, we corrected stem length for shoot dry weight to obtain a relative measure for the stem elongation response to increasing plant density. Both WT and Tetr plants showed an elongation response to increasing plant density (Fig. 2B, Table 1).

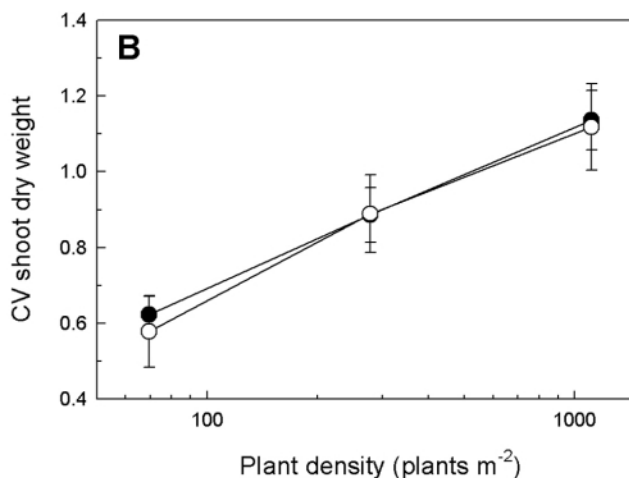
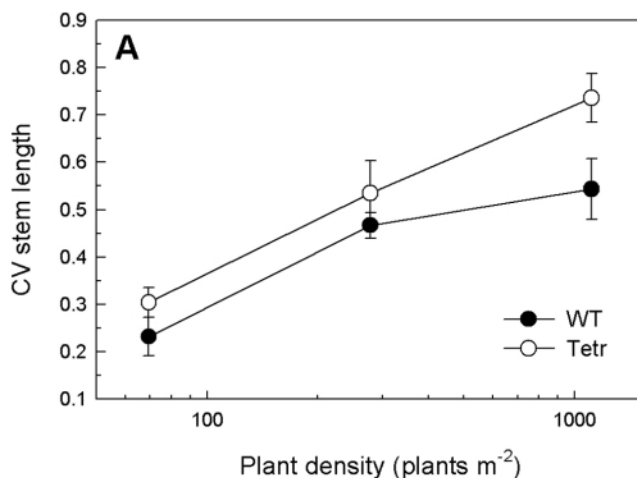
Sharp increases in size inequalities were observed with increasing plant density and, thus, competition intensity (Fig. 3), indicating that size asymmetric competition for light was taking place in both WT and Tetr monocultures. However, the inequality of stem length was significantly lower in WT than in Tetr and this difference seems to be most distinct at the highest density (Fig. 3A), although no significant interaction was found between genotype and plant density (Table 1). CV values of shoot biomass (Fig. 3B) were not different between WT and Tetr (Table 1). Competition for belowground resources was not taking place in our experiment because nutrient supplies were high and whole plot root/shoot ratios decreased with increasing plant density (data not shown), suggesting aboveground rather than belowground competition.

**Table 1** Results of a two-way ANOVA for the selected dependent parameters with genotype and plant density as fixed variables, block as a co-variable and tests for interactions.  $p < 0.05$  indicate significant effects

Variable	Genotype			Density			Interaction		
	df	F value	p	df	F value	p	df	F value	p
LAI	1	0.00	0.9453	2	55.16	0.0000	2	0.13	0.8777
Shoot biomass	1	4.44	0.0460	2	56.57	0.0000	2	0.85	0.4378
Length/biomass	1	3.65	0.0684	2	64.95	0.0000	2	0.07	0.9321
CV length	1	7.17	0.0134	2	27.55	0.0000	2	0.97	0.3924
CV biomass	1	0.07	0.0783	2	17.98	0.0000	2	0.03	0.9639

**Fig. 2** Individual plant shoot biomass (g) (A) and the ratio of stem length and shoot dry weight (cm/g) (B) for WT (solid circles) and Tetr (open circles) tobacco grown in monocultures at 69, 277 and 1111

plants  $m^{-2}$  for 8 wks. Data are means ( $n = 5$  plot means per genotype per density)  $\pm$  SE.

**Fig. 3** Coefficient of Variation (CV) of stem length (A) and shoot dry weight (B) calculated for WT (solid circles) and Tetr (open circles) to-

bacco monocultures grown at 69, 277 and 1111 plants  $m^{-2}$  for 8 wks. Data are means ( $n = 5$  plots per genotype per density)  $\pm$  SE.

## Discussion

Although the total absence of shade avoidance results in severely increased dominance and suppression in plants that have abnormally low growth rates (Ballaré et al., 1994), our results show that a reduced, but not absent, capability of plants

to respond to neighbours does not necessarily result in a large increase in size hierarchies. Furthermore, increased variation in stem length also did not appear to lead to increased size inequalities with regard to shoot biomass.

The two genotypes of tobacco used in the experiments showed increased elongation growth with increasing plant density. Due to the very large overall growth (biomass) reduction at high plant densities, the increased elongation could only be visualised when it was corrected for biomass. After correction, the elongation response increased with plant density, as described in numerous previous publications (e.g., Ballaré et al., 1994; Ballaré, 1999; Weiner and Thomas, 1992). Thus, plants at high densities allocated more to stem growth than plants at lower densities. Wild type plants had more strongly elongated stems than Tetr plants at all densities used (Fig. 2B), which corresponds to reduced shade avoidance responses of ethylene insensitive plants (Pierik et al., 2003).

The reduced change in allometry towards shoot elongation of Tetr plants as compared to WT did not seem to affect light capture, since biomass accumulation was comparable for the two genotypes and identical at the highest density (Fig. 2A). This does not imply that the small manipulation of shade avoidance characteristics in the Tetr transgenic plants has no consequences for competitive outcome at all. Unaffected biomass production compared to WT only occurs if the plants are surrounded by similar neighbours; we previously demonstrated that Tetr plants are severely suppressed when competing with WT neighbours that do respond earlier to neighbouring plants (Pierik et al., 2003). The similar biomasses for WT and Tetr at high plant density suggests that there are no fitness consequences of reduced shade avoidance responses of Tetr compared with the normally responding WT plants in their respective monocultures. This was unexpected since shade avoidance is known to improve light capture in dense stands, thereby enhancing plant fitness (Callaway et al., 2003; Dudley and Schmitt, 1996; Schmitt and Wulff, 1993). There are also indications that enhanced stem elongation in dense stands can actually reduce fitness. However, this only applies to situations where neighbour plants are so large that they cannot be overtopped (Callaway et al., 2003; Maloof et al., 2000). This was demonstrated for *Abutilon* growing in a cornfield, where no individuals were able to overtop the corn (Weinig, 2000), so that the cost (investment in stem elongation) did not provide any gain (increased light capture). That situation, however, does not apply to the present experiment where tobacco plants had only conspecific neighbours of more or less similar size. The fact that plants did overtop each other in our experiment is nicely illustrated by the steep increase in size inequalities (CV) for stem length and biomass with increasing plant density (Fig. 3). This is an obvious indication that competition for light, with increased dominance and suppression, was taking place. Yet, as for shoot biomass, the inequalities for shoot dry weight did not differ between WT and Tetr (Fig. 3B).

Interestingly, size inequalities for shoot dry weight in WT monocultures increased to much higher values (CV from 0.6 to 1.1) in our experiment (Fig. 3B) than in that of Ballaré et al. (1994) (CV from 0.5 to 0.6). WT size inequality of stem length even decreased in their experiment, whereas it increased in ours (Fig. 3A). Although our highest density was two-fold higher than the highest density used in the experiments of Ballaré et al. (1994), the range of LAI values obtained in our experiment were the same as in the experiment of Ballaré et al. (1994), suggesting equal competition intensity. We had therefore expected that our experiment and that of Ballaré et al. (1994) would yield comparable results for WT size inequali-

ties. However, the pronounced density-induced size inequalities we observed appear to be the rule rather than the exception (Aphalo et al., 1999; Miller and Weiner, 1989; Weiner, 1985; Weiner et al., 1990). Contrary to CV values for shoot biomass, inequalities for stem length were higher in Tetr than in WT (Fig. 3A), showing that there is greater variation for stem length in high density Tetr plants than in their WT counterparts. Yet, as discussed above, this increased variation in stem length did not lead to increased inequalities in shoot dry weight. Apparently, the development of (biomass) size inequalities is less sensitive to increased variation in stem length (resulting from reduced shade avoidance responses) than previously thought. This is striking because it is conceivable that plant responses that reduce shading (as in stem elongation in dense stands) should lead to reduced development of size inequalities (Geber, 1989; Schmitt and Wulff, 1993). One explanation may be that, if competition is only allowed for a very short period of time, development of size inequalities may be prevented (Schwinning and Weiner, 1998; Turner and Rabino-witz, 1983), as may have been the case in our experiment. However, size inequalities did increase for both genotypes, suggesting that the duration of the experiment was sufficiently long. Furthermore, plants were harvested just before the onset of flowering at the highest densities, so extension of the growth period would have had no consequences for vegetative growth. It may still be possible that in annual plants, such as tobacco used in the present work, the vegetative phase is so short that it takes very severe disruptions of normal allometric changes upon crowding to significantly affect the development of biomass size hierarchies. This would correspond to the fact that Ballaré et al. (1994) found highly increased density-induced size inequalities in their *PHYA* over-expressing tobacco plants, whereas this was not the case for the Tetr plants in the present work.

We conclude that it requires a severe depression of shade avoidance properties, such as in *PHYA* over-expressing tobacco (Ballaré et al., 1994), to observe significant increases in dominance and suppression at the total shoot biomass level. Since monocultures of ethylene-insensitive Tetr plants had higher CV values than WT for stem length, but not for biomass, we conclude that the development of size inequalities is less sensitive to variation in stem length than anticipated.

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