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Response of yield and physiological attributes to nitrogen availability and heat in maize

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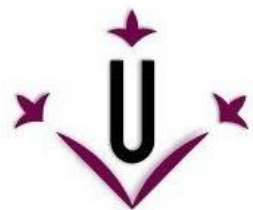
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Response of yield and physiological attributes to nitrogen availability and heat in maize

Doctoral Thesis by

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Dedicatoria

A mis padres Emilio y Elodia

A mis hermanos Silvia, Claudia y Lenin

A la familia Ordóñez Castillo

A Vagi

Por el apoyo y cariño brindado durante mi etapa de formación doctoral.
"Cuando mires atrás y recuerdes tu vida te daras cuenta que la cima no es mas
que perseverancia".

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Abstract

Crop production must be increased substantially in the near future to maintain the present balance with food demand. As a large increase in growing area seems unthinkable, we must further increase crop yields significantly. Climate model projections suggest that higher temperatures will become commonplace in most regions where grain crops are produced, and deleterious effects of high temperature on crop yields are well documented. In this context, it is critical identifying genetic and management tools to mitigate the effect of high temperatures on yield. Nitrogen (N) fertilisation is one of the most widely applied management practices in grain crops worldwide. In many regions, crops are frequently well fertilised to maximise productivity. However, there have been limited efforts to elucidate to what degree the level of soil fertility may affect the magnitude of the high temperature effect on crop yield. Analysing the likely interaction may be relevant for designing more appropriate fertilisation strategies to not only increase productivity through better growth conditions but also to mitigate the likely yield penalties imposed by high temperatures. The general objective of this thesis was to assess the genotypic variability in yield components, and the susceptibility of yield determinants to thermal stress and nitrogen availability in maize. The issue was explored throughout 11 field experiments, carried out during 4 years, at two locations of contrasting altitude, under varying N fertilization regimes and a control with up to 12 different maize hybrids of contrasting maturity groups. In 4 of the field experiments different high temperature stresses were imposed, in combination with genotypes and N regimes, at the field by enclosing the designated area for the treatments with transparent polyethylene film (100 μm thickness) mounted in wood structures of 3-3.5 m height. In several of the experiments source-sink manipulations were also imposed to ascertain the origin of the yield penalties imposed by the different treatments.

Differences in yield performance among hybrids were not related to the cycle duration, however if the comparison is restricted to the average of all short- and long-cycle hybrids, it can be confirmed that the shorter-cycle hybrids had lower production than the long-cycle hybrids. In parallel but independent set of experiments it was found that long cycle hybrids may be a true option for the high altitude farmers (if they are prepared to assume a higher than usual risk of loses in exceptionally cold autumns), as well as the short-cycle hybrids may be a reasonably productive alternative for farmers in the plain of Lleida (and other

similar environments). Overall the range of conditions, yield was more strongly affected by capture, than by partitioning or efficiency of use of resources and was positively related to both of its components similarly (even though grain number was more plastic than grain weight) as well as to grain protein concentration. The negative relationship between yield and Nitrogen Utilisation efficiency (NUtE) found in the context of the wide range of conditions did not preclude the awareness that future hybrids shall be more NU Efficient and that ways to select for improved Nitrogen Use Efficiency (NUE) must be developed for future agricultural systems in which N is expected to be less freely available while yields must keep increased. Then, recently proposed surrogate for phenotyping to improved NUE (the critical specific leaf N, SLNc) was tested for genetic variation. It was proven that large genetic variation exists for SLNc, partly related to genotypic differences in N uptake. This would imply that SLNc would hardly be a good surrogate to phenotype large populations for improved NUE. It was demonstrated for the first time in maize that the sensitivity of yield to heat stress was increased by N fertilisation. This conclusion is based on field experiments with treatments of a magnitude well within expected variation in realistic conditions. The effect was through affecting the capacity of the plants to set grains and to a lesser extent to allow grain weight to be maximised; and it was independent of any (potentially additional) effects on either uncoupling anthesis and silking or on pollen amount and viability. Heat stress affected grain size by directly affecting the capacity of the grains to grow. This conclusion was reached both due to interpretations on the effects of heat on source-sink relationships of plants as well as from results of manipulations of the source-sink relationships during grain filling. Heat stress reduced grain size even when it increased source-sink ratio (by inducing late abortion of few grains while not affecting much post silking growth), and this direct effect was not worsened by defoliation nor reversed by degraining, and the penalty did not exhibit a clear hierarchical response: it was similar for grains of different potential size.

Key words: *Zea mays*, heat stress, NUE, grain number, grain weight, cycle duration, source-sink relationship.

Resumen

En un futuro próximo la producción de los cultivos deberá ser sustancialmente incrementada para poder abastecer la demanda de alimentos. Como futuros aumentos en el área de producción no es posible, la única forma, es aumentar de manera significativa los rendimientos de los cultivos. Las projeccions con modelos climáticos sugieren que la frecuencia de altas temperaturas serán cada vez mayores en la nayoría de las regiones donde se producen cultivos extensivos, y se encuentra muy bien documentado los efectos deletéreos de las altas temperaturas sobre la productividad de los cultivos. En este contexto, será fundamental la identificación de genotipos y esrategias de manejo para mitigar los efectos de altas temperaturas sobre productividad de los cultivos. A nivel mundial, la fertilización nitrogenada (N) es una de las prácticas de manejo ampliamente utilizada en los sistemas agrícolas. En muchas regiones, los cultivos son frecuentemente fertilizados para maximizar la productividad. Sin embargo, han habido pocos esfuerzos para elucidar en qué medida el nivel de fertilidad del suelo puede afectar la magnitud de las altas temperaturas sobre la productividad de los cultivos. El análisis de una posible interacción entre las altas temperaturas y el nivel de nitrógeno en el suelo puede ser relevante para el diseño de estrategias apropiadas de fertilización no sólo para aumentar la producción a través de mejorar las condiciones de crecimiento sino también para mitigar los posibles efectos negativos impuestos por las altas temperaturas. El objetivo general de esta tesis fue evaluar la variabilidad genotípica en los componentes del rendimiento, y la susceptibilidad de los determinantes del rendimiento al estrés térmico combinado con diferentes disponibilidades de N en el cultivo de maíz. El tema fue explorado a lo largo de 11 experimentos en campo llevados a cabo durante 4 años consecutivos y en dos localidades contrastante en altitud, para ello se utilizaron 12 híbridos de maíz de grupos contrastante en madurez bajo diferentes condiciones de fertilización nitrogenada, además de un control sin fertilizar. En 4 de los experimentos de campo, en una combinación de genotipos y regímenes de nitrógeno se impusieron diferentes estreses de altas temperaturas, para ello se cubrió el área designada para los tratamientos con plástico transparente de polietileno (100 μm de espesor) montada en estructuras de madera de 3-3.5 m de altura. Además, en varios experimentos se impusieron diferentes tratamientos de manipulación de fuente-sumidero para determinar el origen de la limitación del rendimiento impuesto por efectos de las elevadas temperaturas.

Las diferencias en rendimiento entre los híbridos no se relacionaron con la duración del ciclo. Sin embargo, si la comparación se limita a la media de los rendimientos observados de todos los híbridos de ciclo corto y largo, se confirma que los híbridos de ciclo corto tuvieron menor producción en comparación con los de ciclo largo. Paralelamente, pero en experimentos independientemente se determinó que los híbridos de ciclo largo pueden ser una verdadera opción para agricultores en condiciones de elevadas alturas tal como en los valles del pirineo (en caso de poder asumir un mayor riesgo que lo habitual de perder la cosecha en otoños con fríos excepcionales), adicionalmente los híbridos de ciclo corto pueden ser una alternativa razonable de producción para agricultores en la plana de Lleida (y otros ambientes similares). En el conjunto de condiciones evaluadas, el rendimiento estuvo mayormente afectado por la captura mas que por la partición o el uso eficiente de los recursos, en los cuales la relación fue positiva y de igual magnitud en ambos componentes del rendimiento (a pesar de que el número de granos fue más plástico que el peso de los granos), al igual que ocurrió con la concentración de proteína en los granos. La relación negativa entre el rendimiento y NUE encontrado en el contexto de la amplia gama de condiciones no se opone a la idea de que los híbridos en el futuro deberán ser más eficientes en NU y que formas de seleccionar para mejorar NUE deberán ser desarrolladas para los sistemas agrícolas futuros en los que se espera que el N será de menor disposición mientras que los rendimientos deben seguir aumentado. Recientemente fue propuesta una alternativa de fenotipeo para mejorar la eficiencia del uso del nitrógeno (el nitrógeno específico crítico en las hojas, SLNc), y esta variable fue utilitzada para evaluar la variabilidad genética. Se determinó que existe una amplia variación genética para SLNc, y parcialmente estuvo relacionado con las diferencias genotípicas en la absorción del nitrógeno. Esto implica que SLNc difícilmente sería una buena alternativa para fenotipeo de grandes poblaciones y mejorar el uso eficiente del nitrógeno. Se demostró por primera vez en esta tesis, que en el cultivo de maíz, la sensibilidad del rendimiento al estrés térmico fue aumentada con la fertilización nitrogenada. Esta conclusión se basa en experimentos de campo con tratamientos con una magnitud de variación similares a las que se esperada en condiciones reales. El efecto fue a través de afectar en las plantas la capacidad para el cuajado de los granos y en menor medida el crecimiento; y esto fue independiente a cualquier (adicional potencial) efecto por el desfase entre la floración masculina y

femenina, o por la disponibilidad y viabilidad del polen. El efecto del estrés térmico en el tamaño potencial de los granos fue directamente a través de afectar su capacidad para crecer. Estas conclusiones se alcanzaron debido tanto a las interpretaciones de los efectos de las altas temperaturas en las relaciones fuente- sumidero propias de las plantas, así como también de resultados de las manipulaciones en la relación fuente-sumidero durante el llenado de grano. El estrés térmico resultó en una reducción del tamaño de los granos incluso cuando se aumentó la relación fuente sumidero (mediante la inducción de aborto tardío de pocos granos sin afectar mucho el crecimiento post-floración), y ese efecto directo no fue empeorado por la defoliación ni revertido por el desgrane, y la penalización no exhibió una respuesta jerarquica clara: fue similar para todos los granos independientemente del tamaño potencial.

Palabras claves: *Zea mays,* estrés térmico, eficiencia en el uso del nitrógeno, número de granos, peso de grano, duración del ciclo, relación fuente-sumidero.

Resum

La producció de cultius ha d'incrementar-se substancialment a curt termini per mantenir l'equilibri amb la demanda alimentaria mundial. Atès que un gran increment de superfície cultivada sembla impensable, l'increment de producció ha de venir per un increment en el rendiment. Els models climàtics suggereixen que temperatures més elevades que les actuals seran habituals en moltes regions del mon on es conreen cultius per a gra, i en aquests, estan ben documentats efectes deleteris degut a les altes temperatures. En aquest escenari, és crític la identificació d'aspectes genetics i de la gestió d'eines per mitigar el efecte de les elevades temperatures sobre el rendiment. La fertilització nitrogenada (N) és una de les pràctiques de maneig més esteses en els cereals de gra arreu del món i en moltes regions els conreus per a gra es fertilitzen bé per a maximitzar la producció. Malgrat això, els esforços per explicar fins a que punt la fertilitat del sòl pot afectar l'efecte de l'elevada temperatura sobre el cultiu son limitats. Analitzar la possible interacció entre la fertilització i les temperatures pot ser rellevant per dissenyar estratègies de fertilització més apropiades no solament per a incrementar la productivitat sino per a mitigar les possibles penalitzacions de les altes temperatures sobre el rendiment. L'objectiu general d'aquesta tesi és l'avaluació de la variabilitat genotípica en els components del rendiment i la susceptibilitat d'aquests components del rendiment al estrès tèrmic de forma combinada amb la disponibilitat de nitrogen en blat de moro. L'estudi es va portar a terme mitjançant 11 experiments de camp, portats a terme durant 4 anys, en dos localitats contrastants en altitud, sota diferents règims de fertilització nitrogenada en 12 diferents híbrids de blat de moro de diferents grups de maduració. En 4 dels experiments de camp es van imposar diferents estresses de temperatures elevades en combinació amb diferents hibrids de panis i diferents règims de nitrogen, mitjançant l'envoltament d'una zona determinada amb estructures de fusta de 3-3,5m d'altura cobertes amb polietilè transparent (100 micres). En varis dels experiments es van realitzar també manipulacions font-embornall de manera factorial per establir l'origen de las penalitzacions en rendiment dels diferents tractaments. Les diferències en la resposta del rendiment dels diferents híbrids no van estar relacionades amb la duració del cicle del híbrid, no obstant això si la comparació es restringeix a la mitjana de tots els híbrids de cicle- curt i els de cicle-llarg, els híbrids de cicle-curt van tenir produccions més baixes que els de cicle-llarg. En paral·lel, però en un grup d'experiments

independent és va observar que els híbrids de cicle (relativament)-llarg podrien ser una bona opció pels agricultors que cultiven en altituds elevades (si estan preparats per assumir un risc més elevat del habitual de pèrdues degudes a tardors excepcionalment fredes). Així mateix, els híbrids de cicle-curt podrien ser una alternativa de producció raonable per pagesos en la plana de Lleida (i altres ambients similars). En general pel conjunt de les condicions estudiades, el rendiment de gra va resultar estar més afectat per la captura de los recursos (aigua, N, etc.) que pel seu repartiment o per l'ús eficient dels mateixos, i va ser positivament relacionat amb ambdós components de rendiment amb igual magnitud (encara que el numero de grans va ser més plàstic que el pes del grans) així com amb la concentració de proteïna al gra. La relació negativa entre el rendiment i l'eficiència del l'ús del nitrogen (*nitrogen use efficiency,* NUE) trobada en aquest context d'ample varietat de condicions, no pot descartar el fet de que híbrids futurs haurien de ser més eficients en l'ús del nitrogen. Per tant cal desenvolupar noves eines de selecció per millorar NUE pels futurs híbrids i sistemes agrícoles en els que es preveu que el N estarà menys disponible, al mateix temps que s'ha de continuar incrementant el rendiment. Un estudi recent va proposar l'estudi del contingut específic de nitrogen en fulla (*critical Specific Leaf Nitrogen*, SLNc). Aquest estudi va demostrar que existeix una gran variabilitat genètica per SLNc, en part relacionada amb diferencies genètiques en l'absorció de N. Aquest implicaria que el SNLc difícilment podria ser una bona alternativa per l'estudi del fenotip de grans poblacions per millorar l'eficiència del l'ús del nitrogen. En aquesta tesi s'ha demostrat per primera vegada en blat de moro que la sensibilitat del rendiment al estrès tèrmic és va incrementar amb la fertilització nitrogenada. Aquesta conclusió està basada en experiments de camp amb condicions realistes, reflectint de manera molt similar les variacions ambientals esperades (tèrmiques, aigua, N, etc.). L'efecte es va observar en la capacitat de les plantes per establir grans i en menor grau en permetre maximitzar el pes del gra i va ser independent de qualsevol efecte potencial del acoblament en la fecundació (sortida de sedes – alliberació del polen) o en la quantitat i viabilitat del pol·len. L'estrès tèrmic va afectar la grandària del gra afectant directament la seva capacitat de creixement. A aquesta conclusió s'hi va arribar a partir de les interpretacions del efecte de temperatures elevades sobre les relacions font-embornall de les plantes i dels resultats de les manipulacions de las relacions font-embornall durant l'etapa d'omplenament del gra. L'estrès tèrmic va reduir la grandària del

gra inclús quan es va incrementar la relació font-embornall (induint la aborció tardana dels pocs grans al temps que no s'afecta el creixement després de la sortida de sedes) i aquest efecte directe no va ser empitjorat ni per la defoliació ni per el desgranat i la penalització no va mostrar cap resposta jeràrquica clara: va ser similar en grans amb diferent grandària potencial.

Paraules claues: *Zea mays,* estrès tèrmic, eficiència del l'ús del nitrogen, numero de grans, pes de grans, duració del cicle, relació font-embornall

Chapter I

General Introduction

1.1 Trends in population growth, climate change and crop production

Current trends in population growth suggest that global food production is unlikely to satisfy future demand under predicted climate change scenarios unless the rates of crop improvement are accelerated or radical changes occur in the patterns of human food consumption (Reynolds et al. 2011). Just three staple crops, wheat, maize, and rice, provide approximately 50% of the calories and 42% of the protein for human consumption in less developed countries (Braun et al., 2010).

Based on projection models it has been estimated that by 2025 the population will increase more than 1.5 billion (Lutz et al., 2001) and at the middle of the century the population will reach around 9 billion (Godfray et al., 2010). Looking forward in 2050, to meet the increased demand for grain and to feed the growing population on the available arable land, is suggested that annual crop production should be increased (Ray et al., 2013). Thus, is predictable that food production has to increase by at least 70% before 2050 in order to support the continuous growing population (Parry and Hawkesford, 2012). In addition, population growth is expected to result in a doubling of demands of food from livestock and agricultural land (Byrnes and Bumb, 1998; Naylor et al., 2005).

Increases in crop production will necessary arrive from increases in yield as the amount of cropping land will not be increased (Albajes et al., 2013; Fischer et al., 2014). Further increases in crop production will come from improvements in yield potential or by reducing the gap between actual and potential yield, throughout better management practices.

On the other hand, all over the world, crop production will become more difficult due to climate changes, resource scarcities (*e.g.* land, water, energy and nutrients) and environmental degradation (*e.g.* declining soil quality, increasing greenhouse gas emission, and surface water eutrophication). The effect magnitude will depend on the future scenario of CO_2 predictive increases and other atmospheric gases as well the world economic activity. According to IPCC (2007) the global mean surface temperature will increase in the near future by 1.1 to 6.4 ºC at the end of the century. So as the global temperature increases, the frequency of extreme high temperatures will also increase multiplying the frequency of heat shock appearance (Tebaldi et al., 2006).

Under this scenario, understanding the physiological basis of crop yield determination under different management practices will be crucial in every cropping region in order to achieve higher yields.

1.2 Importance of maize and yield determination

Maize (*Zea Mays* L.) is one of the most important grain crops. Even though, maize was originated from Mesoamerica, nowadays is the most widely cultivated cereal crop. Maize genetic diversity plays an important role in the distribution of the crop in different ecological zones; from the sea level to more than 3000 m highland temperate environment (Fig. 1a) (Buckler et al., 2009). In developing countries, in America and Africa, white maize grain is mainly used for human consumption (Prasanna, 2012; Shiferaw et al., 2011). In addition, maize is an excellent source of starch feed for livestock (Hellin et al., 2013; Herrero et al., 2010) and also for bioethanol and biofuel production (Dunwell, 2000; Mackay, 2009; Torney et al., 2007).

The average of the total maize production around the world during the last decade was around 872 million of Mg, where the top five production countries were United States, China, Brazil, México and Argentina. Spain is listed 17th in the production countries with 4.3 million of Mg (Fig. 1b) (FAOSTAT, 2014).
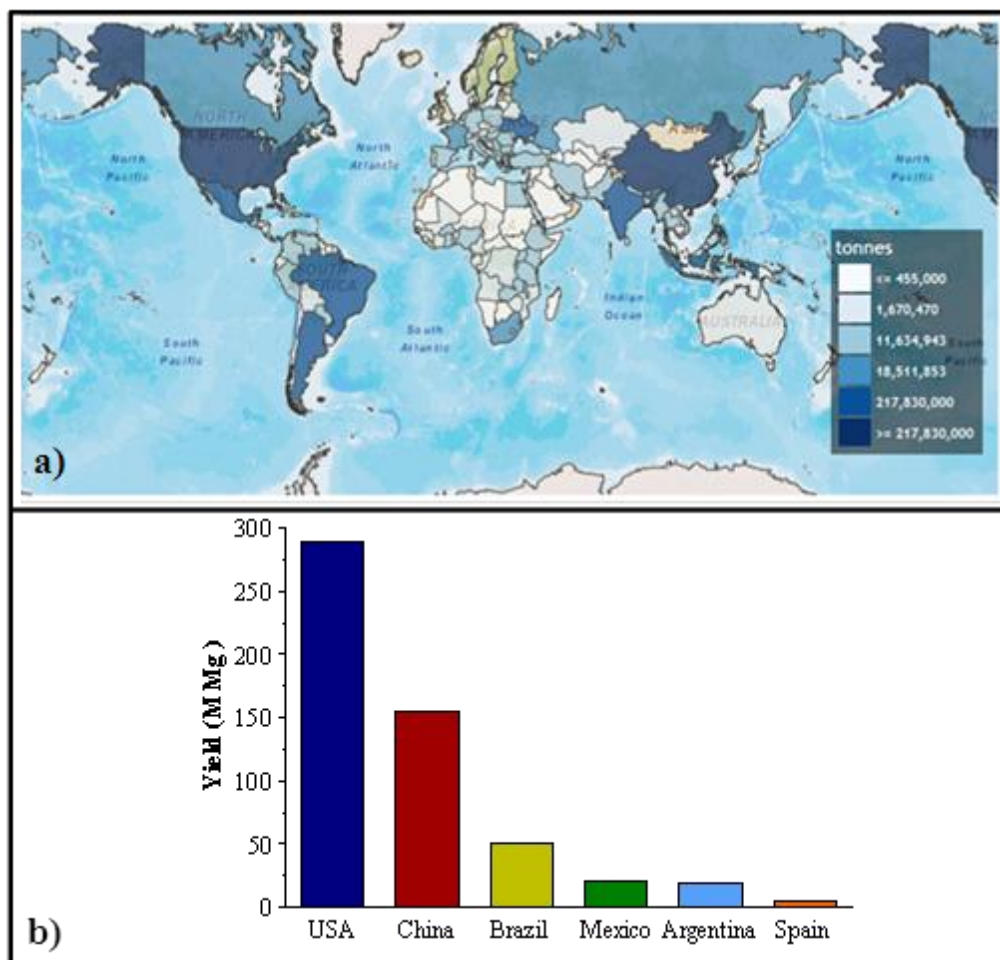
Figure 1. World yield maize production; a) actual maize yield production around the world in Millions of tonnes, (2013), b) the top five countries in maize production based on averaged data yield registered from 2000-2013. Source: FAOSTAT

Yield in maize is the consequence of the interaction between the number of grains and their average weight. Grain number is mainly determined during the critical period of 30 days bracketing silking (Andrade et al., 1999; Vega et al., 2001; Andrade et al., 2002; Westgate et al., 2004) when the number of grains per plant is determined in line with the rate of growth of the crop during that period (Vega et al., 2001) because it is during this period when the juvenile ear, where the female florets are developing, grow (Otegui and Bonhomme, 1998) and then the abortion process affects a proportion of the pollinated florets. Grain weight potential is largely determined during the same period (Gambín et al., 2006) and formally realised during the "lag phase" (Maddonni et al., 1998); but final grain weight is realised during the effective period of grain filling (Cirilo and Andrade, 1996; Borrás and Otegui, 2001).

1.3 Role of nitrogen in yield productivity and its interaction with heat stress

Nitrogen is considered one of the most important factors determining crop production (Follett and Hatfield, 2001; Hall et al., 1982; Parry et al., 2005). Nitrogen fertilisation is unquestionably one of the most widely applied management practices in grain crops worldwide. In many regions, crops are frequently well fertilised to maximise productivity.

The consumption of nitrogen has been increased in about 150% for many crops. This tremendous increase was observed in the last 30 years when nitrogen started to be used intensively in agriculture (Frink et al., 1999). However, substantial proportion of applied nitrogen is lost by nitrate leaching, denitrification and loss of ammonia to the atmosphere having serious impact in the environment (Cameron et al., 2013; Francis et al., 1993; Glass, 2003). For this reason, improvement in nitrogen use efficiency (NUE) is a major task in most breeding programs.

A major avenue to increase NUE is the genetic improvement of this attribute. As many thing in life the problem is that it is much simpler to say than to do this. NUE is extremely complex physiologically and in practice almost impossible to measure in realistic breeding programs. For that reason, different authors have been trying to identify ways to phenotype for improved NUE. Substantial advances have been made in methodologies for phenotyping complex traits in maize breeding (Araus and Cairns, 2014), and many phenotyping tools have been identified (Cairns et al., 2012). Around silking, when yield potential is being determined (Munaro et al., 2011; Paponov et al., 2005; Tollenaar et al., 1992), most plant N is allocated to leaves (Muchow, 1988; Tsai et al., 1991; van Oosterom et al., 2010). Then, for maximizing NUE leaf N at silking must be considered. Several papers have highlighted the critical role of specific leaf N (SLN, the mass of N per unit of leaf area) in maximizing crop growth during the critical period of silking, and thereby in maximizing yield (DeBruin et al., 2013; Massignam et al., 2011; Muchow and Sinclair, 1994; Sadras et al., 2000). One side aim of this thesis has been to test one recently proposed alternative for phenotyping to improve NUE in maize.

Yield of cereals is impaired by higher temperatures during the growing season (Hatfield et al., 2011), but particularly when they occur during the most critical periods of yield determination. High-temperature effects on yield may affect grain number and grain

weight, depending on the timing of occurrence of the heat (Rattalino et al., 2013). In principle, if the penalty imposed by the heat stress operates, at least partly, through reductions in crop growth, yield would be more affected when the heat occurs in the grain number determination period (around silking) as grain number determination is clearly source-limited (Gambín et al., 2006; Slafer and Savin, 2006) whilst grain weight seems more limited by the sink strengths (Gambín et al., 2008), at least if severe defoliations or very low levels of incoming radiation do not occur during the effective period of grain filling (Borrás et al., 2004). If the effects were not mediated by reducing crop growth, the magnitude of the penalty would be similar whether the stress occurs around silking or during the effective period of grain filling. It seems likely to hypothesise that high-temperature effects may be indirect, mediated by reducing crop growth (e.g. Cicchino et al., 2010b), though direct effects not mediated by reductions in growth are possible (Rattalino and Otegui, 2013).

A major inconvenience of studies aimed to uncover high-temperature effects on crop productivity is that, due to the difficulties in imposing the treatments under field conditions, they are most frequently conducted under controlled conditions. These studies are extremely useful for understanding detailed mechanisms of action of particular factors at relatively low levels of organisation. The problem is that results can hardly be extrapolated to field conditions (Passioura, 2006), where the practical consequences are expected. Scaling up from controlled conditions experiments to application in realistic field conditions may present several constraints (Passioura, 2010).

Recently a number of studies were conducted in the experimental field of the Universidad of Buenos Aires by the group of Prof. Otegui enclosing for particular periods the maize canopy with transparent polyethylene film mounted wood structures build up *a priori* (Cicchino et al., 2010a; Rattalino and Otegui, 2011). A step forward in direction to increase the actual value of the conclusions to realistic system is to run such experiment in farmer fields and in interaction with very common management practices, such as nitrogen fertilisation.

Several agronomic and genetic strategies for increased tolerance to high temperatures will be necessary (Rosenzweig et al., 1994). The likelihood of mitigations through using plant growth regulators (Cicchino et al., 2013), or adequate management of magnesium

(Mengutay et al., 2013) are being discussed. Around the world, food production increased linearly with the increment of nitrogen use in the agricultural systems (Tilman 1999), and nitrogen fertilizacion is likely the most common management practice implemented in maize production worldwide. High yields in maize crop are closely associated with nitrogen fertilization (Setiyono et al., 2010), mainly through affecting grain number (Carcova et al., 2000; Paponov et al., 2005) through modifying crop growth during the critical period around silking (Andrade et al., 2002; D'Andrea et al., 2008).

To the best of our knowledge, the interaction between heat stress and nitrogen availability has not been tested in maize. Both in wheat (Altenbach et al., 2003; Zahedi et al., 2004) and in barley (Passarella et al., 2008) it has been shown that the penalty on yield imposed by exposure to high temperatures were affected by the level of nitrogen availability: the higher the availability the more damaging the high-temperature effect (Altenbach et al., 2003; Zahedi et al., 2004; Passarella et al., 2008). If a similar sort of interaction were demonstrated, it may have relevant practical implications as in the future, when maize would be more often exposed to heat stresses, decisions on rates of nitrogen fertilisation should be taken not only considering the potential beneficial effects on crop growth but also a potential trade off on the magnitude of the penalty produced by heat stresses.

1.4 Regional characterisation

In the case of the province of Lleida (Catalonia, NE Spain), under irrigation condition, the maize crop is sown from the region of the Plain to the valleys of the Pyrenees, along an altitudinal gradient. Most often in the region of the Plain farmers used long-cycle hybrids, and that would maximize yield potential although occupy the field for longer, while in the valleys of the Pyrenees traditionally short-cycleare used because the period of grain filling occurs at lower temperatures. While this premise is eminently logical (with increasing altitude tends to grow shorter cycle hybrids, probably sacrificing yield potential but reducing the risk of loss of actual performance by insufficient grain filling under conditions of rapid drop in temperatures). However, in the Plain region would be valuable growing short cycle hybrids, either as an alternative to a maize planted very late in succession to another crop in the same growing season (*e.g*. barley or a legume),

if the reduction performance over traditional long cycles were not greater than the benefit of the previous crop. In the region of the Pyrenees, in turn, could be useful to know the behavior of modern long-cycle hybrids that eventually could express their greatest potential in terms of radiation-temperature relationships. Comparative studies of the behavior of contrasting cycle hybrids are not common in these regions.

1.5 Objectives

The general objective of this thesis was to assess the genotypic variability in yield components and the susceptibility of yield determinants to thermal stress and nitrogen availability in maize.

Different specific objectives were outlined:

(i) To determine genotypic differences in the physiological determinants of yield and also to identify physiological traits associated to the genetic variation in maize hybrids differing in their developmental cycle (Chapter II).

(ii) To analyze the responsiveness of senescence traits and yield components in a long- and a short-cycle maize hybrids (chosen from the previous experiment, Chapter II) at locations of contrasting altitude and under low and high N availabilities (Chapter III).

(iii) To determine the degree of genetic variation, if any, in a range of modern maize hybrids grown under contrasting growing conditions able to generate a wide range of both yield and SLN values for each hybrid (Chapter IV)

(iv) To explore the magnitude of yield penalty that is imposed by high temperature depending on whether the stress occurred around flowering or during early grain filling and on the availability of nitrogen (Chapter V).

(v) To study if the differences in grain weight reductions are due to high temperature during grain filling and whether it operates directly on the capacity of the grains to grow rather than increasing the competition for limited assimilates (accelerating senescence) by the grains (Chapter VI).

1.6 Outline of the present thesis

This thesis is divided into eight chapters. The chapters included a general introduction (Chapter I), five experimental chapters and a global discussion (Chapter VII) and conclusion of the entire thesis (Chapter VIII).

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Chapter II

**Variation in yield generation within modern maize hybrids of
different maturity type under contrasting growing conditions**

2.1 Introduction

World population growth demands an increase in food productivity. As the growing area could hardly be increased (*e.g.* Albajes et al., 2013 and references quoted therein), satisfaction of this increased demand must be produced by increases in yield (Fischer et al., 2014). Moreover, climatic change and extreme weather events will exacerbate the fragile of food production systems, especially in places affected by soil degradation, water stress, and high temperatures (Rosegrant and Cline, 2003)

Increases in yield may come from improvements in yield potential or by reducing the gap between actual and potential yield, by designing the most appropriate strategies to increases grain yield of a specific cultivar (Andrade et al., 2005). In irrigated intensive systems (such as those prevailing in Catalonia) this gap is small and yield potential must be further increased.

In Catalonia there are two distinct areas of maize production: the plain and the Pyrenees which are characterized by using hybrids of different cycle length, commonly hybrids sown at the plain are longer than the ones sown at the Pyrenees.

In general, it is accepted for field crops that the longer the cycle, the higher the yield (Richards, 1996), because the longer the season, the more resources (radiation, water and nutrients) will be absorbed by the crops. However, yield is particularly sensitive to growth and partitioning during a critical period (Andrade et al., 1999; Vega et al., 2001; Slafer and Savin, 2006) and this would make it difficult to directly accept the linear relationship between crop cycle and yield potential, if the cycle does not affect growth in the critical period. Grain yield in tropical environments was demonstrated to be lower than in temperate environments (Muchow et al., 1990). The differences between both environments were due to an increase in the duration from emergence to tassel initiation and to final leaf number (Birch et al., 1998), resulting in an increase in total biomass. Additionally, under contrasting temperatures and cycle duration, the long-cycle hybrid productivity was reduced by the frost temperature during filling grain (Wilson et al., 1994), which is common at the Pyrenees during grain filling period in maize. Capristo et al. (2007), working at the maize belt region in Argentina, found yield increases with increasing length of the growing cycle to a threshold beyond which performance is stabilized. These results suggest that, within what might be generically termed intermediate- and long-cycle hybrids; yield would be relatively independent of the duration of the cycle. Longer cycle hybrids presented longer duration than the

intermediate ones, but these longer stages occurred in less favorable conditions (*e.g.* lower temperatures, which can affect crop radiation efficiency, Andrade et al, 1992). Furthermore, short-cycle hybrids generally have a lower-yielding performance due to lower radiation interception during the critical period; lower harvest index; and a shorter duration of the different phenological phases (Capristo et al., 2007). In particular agronomic system, there may be circumstances in which it is favorable to use different cycle hybrids. Bruns and Abbas (2005), for the area of Mississippi (USA), argued that the use of short-season hybrids could be beneficial in an environment of high temperatures and drought that increase the likelihood of contamination by mycotoxins. Even in growing conditions that pose no risk of toxicity according to the moment maturity, the use of short-cycle maize genotypes might be advantageous in some productive scenarios. These hybrids enable an earlier harvest with avoiding high humidity (DAR, 2008) and may also be sown much later without increasing considerably the risk of yield losses due to early frosts.

In the case of the province of Lleida (Catalonia, NE Spain), the maize crop is sown from the region of the plain to the valleys of the Pyrenees, along an altitudinal gradient. Most often in the region of the plain farmers used long-cycle hybrids, and that would maximize yield potential although occupy the field for longer, while in the valleys of the Pyrenees traditionally short-cycle are used because the period of grain filling occurs at lower temperatures. While this premise is eminently logical (with increasing altitude tends to grow shorter cycle hybrids, probably sacrificing yield potential but reducing the risk of loss by insufficient grain filling under conditions of rapid decline in temperatures). However, in the plain region would be valuable growing short cycle hybrids, either as an alternative to a traditional maize but planted much later allowing to harvest another grain crop in the same growing season (*e.g.* wheat, barley or a grain legume), if the reduction performance over traditional long cycles were not greater than the benefit of the additional crop. In the region of the Pyrenees, in turn, it could be useful to know the behavior of modern long-cycle hybrids that eventually could express their greatest potential in terms of radiation-temperature relationships. Comparative studies of the behavior of contrasting cycle hybrids are not common in these regions.

Both in the Pyrenees and in the plain, farmers use to fertilise maize crops with high doses of nitrogen (N) (*c*. 300 KgN ha^{-1}), under soils that many times have already accumulated important amounts of this nutrient (Cela et al., 2011), so it is likely that

with half of the doses maximum yields may be achieved (Berenguer et al., 2009). Due to the serious contamination problems from excessive fertilisation, it would be expected that in the future N will be less abundantly used. Thus, it is interesting to evaluate the behavior of modern hybrids of different cycle in contrasting conditions of N availability.

In the present study we screened a number of commercial, well adapted, hybrids of a wide range of maturity types (as classified commercially) to determine (i) their performance under environments close to Algerri (a relatively warm environment in the Ebro Valley) and close to La Seu d'Urgell (a relatively cold environment in high altitude valleys of the Pyrenees) under contrasting N fertilisation regimes, and (ii) whether the performance of two of them, used in more in depth analysis in parallel experiments (Chapter III), could be considered reasonably representative of other commercially relevant hybrids in the region.

2.2 Material and methods

2.2.1. General conditions

Four field experiments were carried out to compare the performance of 12 different maturity type hybrids under two contrasting N availabilities. The experiments were conducted at two locations differing in altitude (Fig. 1) being one then representative of relatively warm environments and the other of relatively cool environments of maize production at Catalonia (NE Spain). The warm environment was a location in the plain of the province of Lleida close to Algerri (41º47´41´´ N; 0º38´52´´ E, 230 m alt) and the cool environment was a field in a valley in the middle of the Pyrenees close to La Seu d`Urgell (42º 20' 40.6" N; 1º 25' 47.4" E; 730 m alt). In both locations experiments were carried out during two consecutive experimental years, 2009 and 2010. The initial conditions and soil properties are shown in Table 1.

Figure 1. General view of the experiments in the Pyrenees (upper picture) and the plain of Lleida (lower picture).

Table 1. Main soil properties before sowing in each location.

Soil properties	Location			
	2009		2010	
	Plain	Pyrenees	Plain	Pyrenees
Sowing date	12-May	11-May	17-May	20-May
Sowing rate (plants ha^{-1})	80,025	85,000	84,030	85,000
N-NO3- (kgN ha^{-1})	175¥	150‡	141¥	129‡
Phosphorus Olsen (kgN ha^{-1})*	70	88.2	35.1	70
pH (Ext.1:2:5 H2O)*	8.2	8	8.2	8
EC 25ºC (Ext. 1:2.5 H2O) dS/m*	0.4	0.1	0.2	0.1
Organic Matter (Walkley-Black, %)*	1.9	1.4	1.5	1.4
USDA textural soil classification)	Clay loam	Sandy loam	Clay loam	Sandy loam

Soil samples taken from *0-30 cm, ‡0-75 cm, ¥0-100 cm depth

The plain of Lleida is characterized by a dry continental Mediterranean climate with average rainfall of *c*. 400 mm and average temperature of 15 $^{\circ}$C during the year; while at the Pyrenees of Catalonia the annual average rainfall is *c*. 750 mm and the average temperature of 11 $^{\circ}$C (Meteorological Service of Catalonia 2011). Daily global radiation, minimum and maximum temperature, and precipitation during the

experimental years were recorded at standard meteorological stations of the Agro-meteorological network of Catalonia, Spain, located close to the experimental fields (Fig. 2).
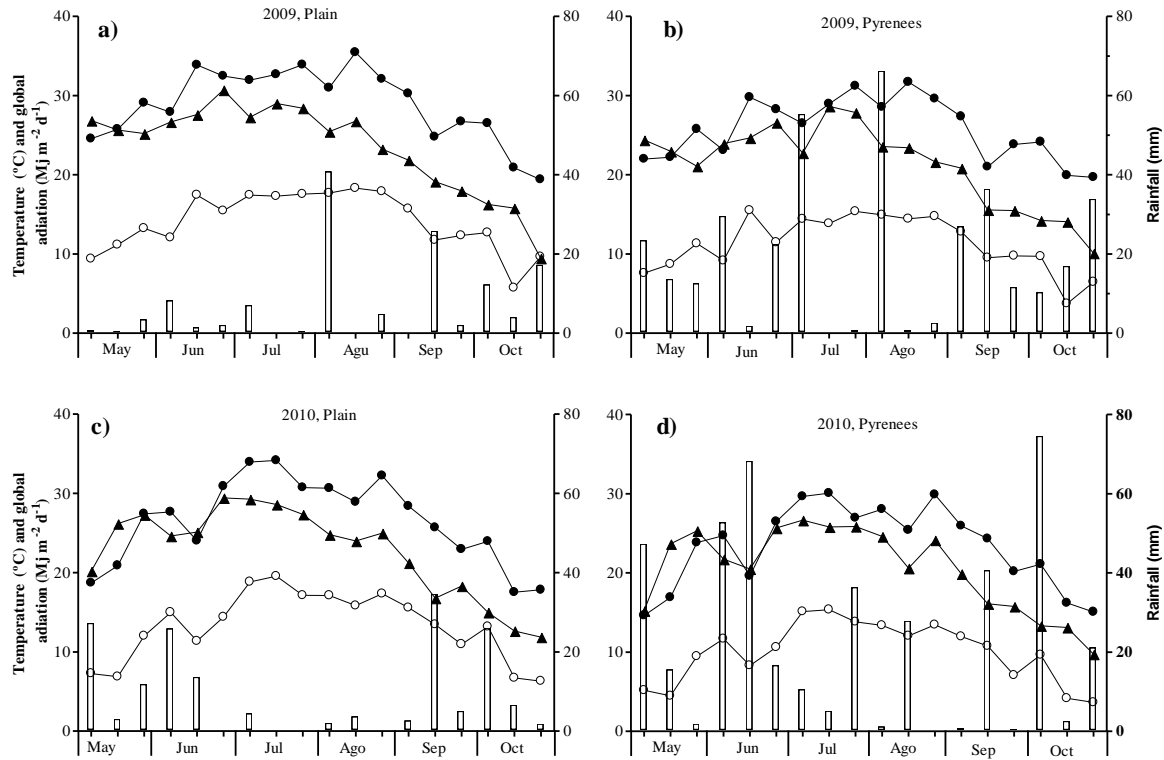
Figure 2. Mean global radiation (triangles), maximum temperature (closed circles) and minimum temperature (open circles), and accumulated rainfall (open bars) for each 10-d interval from sowing to maturity in field experiments carried out during 2009 and 2010 at the plain of Lleida (a, c) and Seu d`Urgell (b, d).

Experiments were sown within normal sowing periods of the different zones, and were well irrigated (near field capacity throughout the growing season) with sprinkler irrigation in La Seu d'Urgell and also in the first year in the plain and with drip irrigation in the second season of the plain. All the experiments were always kept free of weeds, pest and diseases by spraying recommended herbicides, insecticides and fungicides at the doses suggested by their manufacturers whenever was necessary.

2.2.2 Treatments and design

All experiments were conducted in typical field conditions. Actually the experiments were carried out in real farmers' fields we rented for this purpose and all the equipment and practices were those used by the farmers, with the exception of the N fertilisation regimes and hybrids, which were the treatments of the present study. Thus, treatments included a factorial combination of 12 maize hybrids and two nitrogen levels. Farmers in the plain of Lleida use most frequently long cycle hybrids. On the other hand, in the Pyrenees short cycle hybrids are grown almost exclusively. The selection of these hybrids was based on the contrasting maturity cycle but restricting the selection to those representing commercial hybrids (with high yield potential adapted to these regions; Table 2).

Regarding fertilization regimes, two contrasting treatments were used: a control (not additional nitrogen applied to the soil, N0) and a treatment fertilised with 200 kgN ha^{-1} (N200) in which urea was broadcasted manually at V6 (when the sixth leaf was expanded; Richie and Hanway, 1982), usually close to an irrigation to warrant quick incorporation to soil.

In the plain of Lleida, the main plot consisted of 8 rows x 20 m length with a distance of 0.70 m between rows, having in both sites a final plant density of ~8.4 plants m^{-2}. In the Pyrenees the main plot consisted of 15 m length with a distance between rows of 0.75m; the distance between plants was changing in order to achieve a plant density ~8.4 m^{2}. All experiments were arranged in a split-plot design with three replications where hybrids were the main plots and N fertilisation regimes were the sub-plots

Table 2. List of the 12 maize hybrids sown in the experiments carried out at the plain and the Pyrenees during 2009 and 2010.

Genotype		Origin company	Growth cycle (FAO Classification)
Number	**Name**		
1	Franki	Caussade Semences	400
2	Eurostar	Euralis	400
3	Earlystar	Euralis	400
4	Lapopi	Caussade Semences	450
5	Klimt	KWS	600
6	Beles Sur Bt	Limagrain	650
7	Paolis	Caussade Semences	650
8	PRN31N28	Pioneer	700
9	DKC6575	Monsanto	700
10	Guadiana	Limagrain	700
11	Kermes	KWS	700
12	Helen Bt	Limagrain	750

2.2.3 Sampling and measurements

In each experimental unit we determined the timing of anthesis and silking when the plants in that unit were shedding pollen from the tassels and emerging stigmas (silks) from the husks of the ear, respectively. The timing of maturity was determined by periodic inspection at the end of the grain filling period when the black layer was formed. When the duration of developmental phases was expressed in thermal time, we used a base temperature of 8ºC (Cirilo and Andrade, 1996).

Plant samples (two rows, 1 m long; *i.e.* 1.4 m^2 including *c.* 11-12 plants) were taken for each experimental unit site at silking and maturity. Dry weight and N content (Kjeldahl) was determined for different plant tissues (stem, leaf blades, small ear and grains). At maturity, grains were separated and weighted and counted to determine final grain number and average grain weight.

All data were analyzed by ANOVA to evaluate the effects of treatments and their interactions. A t-test was used to determine significant differences ($P < 0.05$; <0.01; <0.001) between means. The relationship between variables was analysed by linear regression. A multivariate analysis of principal components was performed to the main variables and for all treatments (12 hybrids, 2 locations, 2 years and 2 N availabilities).

2.3 Results

2.3.1. Crop phenology

As there was no interaction between years and duration to silking or maturity in the 12 hybrids, these results are presented as and average for the two years. The duration explored from sowing to silking stage in the plain was from 820 to 1050 °C d (59 and 73 days), and from 760 to 913 °C d (69 to 80 days) in the Pyrenees (Fig. 3). In time duration from emergence to silking stage, significant differences were observed between the hybrids (Fig. 3).

Selection of hybrids was based according to FAO classification, from FAO 400 to FAO 750 types (Table 2), expecting a continuous variation in their phenology. However, in each environment tested, there variation was actually mainly discrete: there were two groups according to phenological data, one group of 4 hybrids that we will call from now on the short-cycle hybrids while the other hybrids will be called the long-cycle hybrids. The short cycle group were the ones that showed less thermal time from sowing to silking (Franki, Eurostar, Lapopi and Earlystar), and the long cycle group were integrated by the rest of the hybrids (Fig. 3).

In the plain of Lleida, grain filling duration tended to be reduced in the short-cycles in comparison with the long-cycle hybrids, but not always (*e.g*. Lapopi time to silking was similar to that period of other short-cycle hybrids, but it was similar to a long-cycle during grain filling; Fig. 3). In the Pyrenees, there was no relationship between the timing from silking to maturity and the FAO classification of the hybrids (Fig. 3).
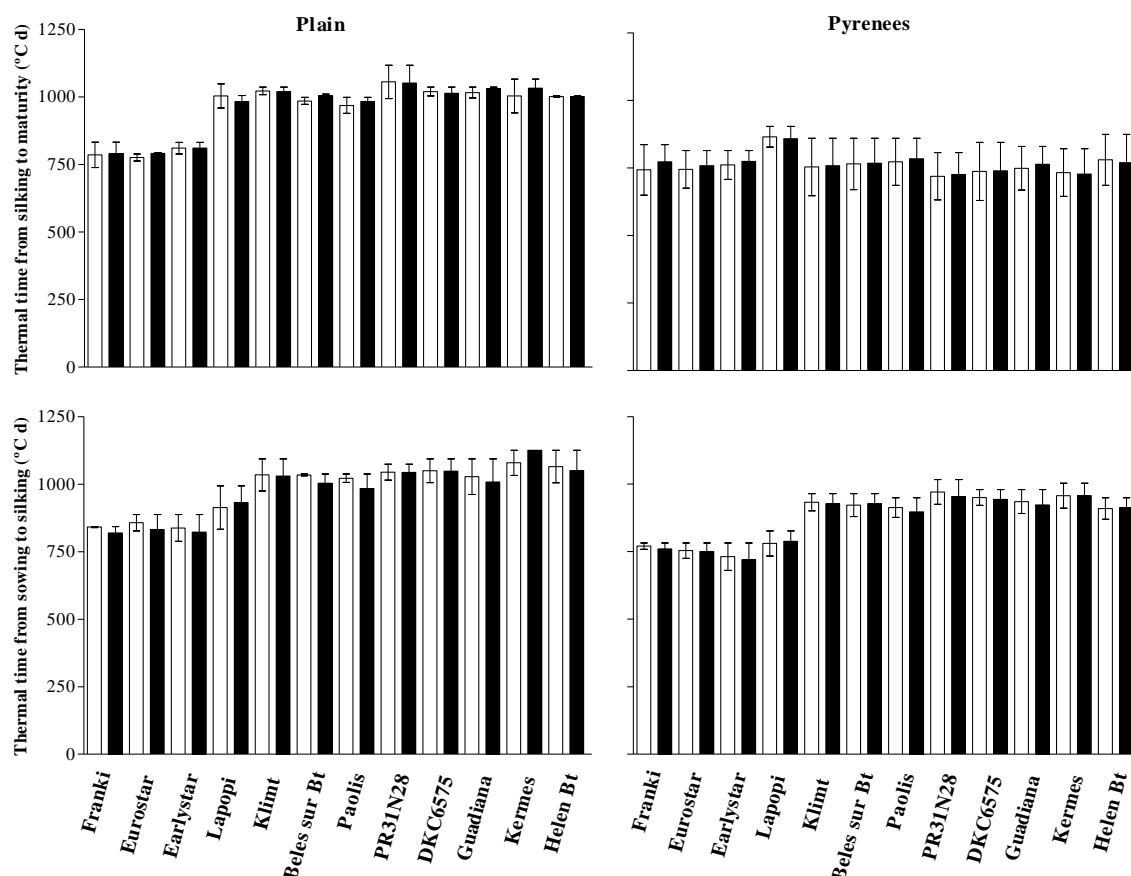
Figure 3. Thermal time from sowing to silking (bottom panels) and silking to maturity (top panels) in average for the two years (2009 and 2010) for the 12 hybrids (ordered according with FAO classification; Table 2) at the plain of Lleida (left panels) and the Pyrenees (right panels) under different N availabilities (N0=open bars; N200=black bars). Segments stand for the standard error of the means.

Duration of the phenological phases (which considering the two phases from sowing to silking as well as the duration of grain filling, the 12 hybrids, the two locations and the two years amounted 96 cases in which N effects were explored) were not significantly altered by fertilisation (Fig. 3). There could be particular combinations of hybrids x locations x years x phases, in which development differences in response to N fertilisation were occasionally apparent; but they were not only statistically not significant but also small in absolute terms, erratic in direction (either increasing or decreasing duration when fertilised), and not consistent (between locations or years).

2.3.2 Grain yield

Grain yield varied from *c.* 6 to *c.* 17 Mg ha^{-1}; considering experimental years, locations, N availability and the 12 hybrids (Fig. 4).
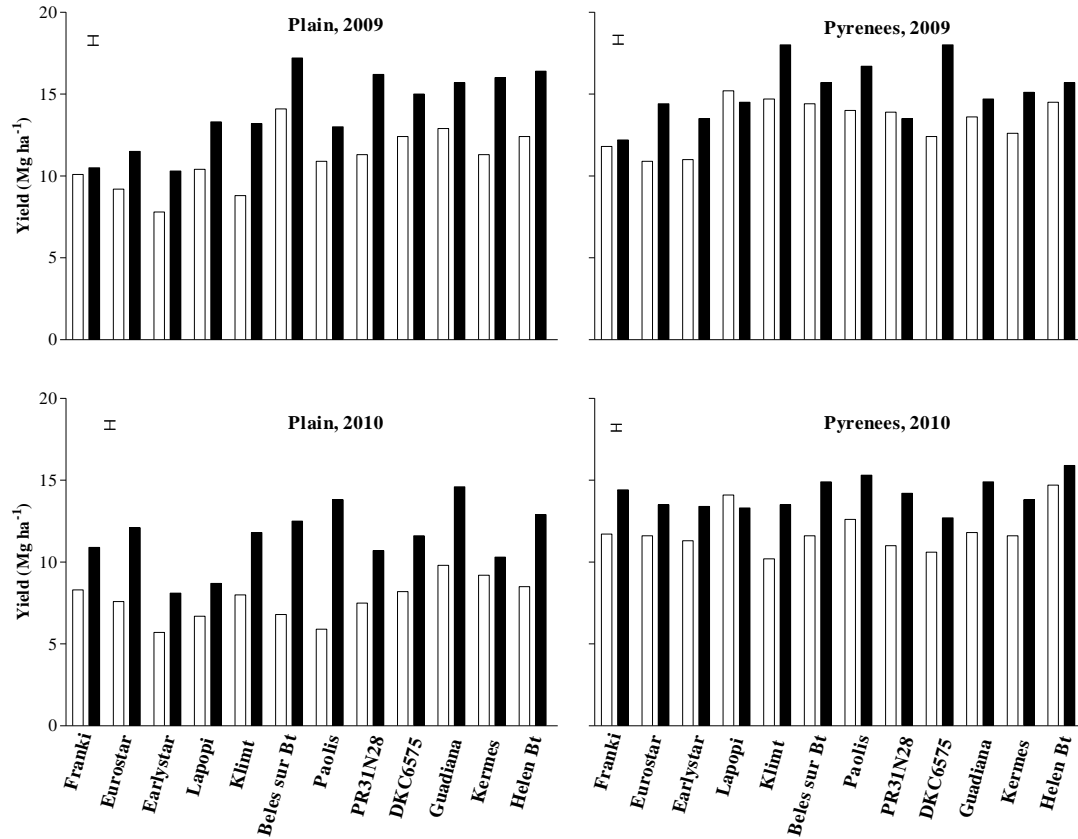
Figure 4. Grain yield for each maize hybrid (ordered according with FAO classification; Table 2) during two experimental years (upper panels for 2009 and lower panels for 2010). Open bars represents the unfertilised control and the closed bars the fertilised treatment. The segments in each panel represent standard error of the means of the corresponding ANOVA.

Grain yield was higher in 2009 than in 2010 (13.4±0.3 *vs* 11.3 ±0.4, respectively) and in the Pyrenees than in the Plain (13.6±0.3 *vs* 11.0 ±0.4, respectively). Apparently, observed yield for each hybrid in the Pyrenees was higher than the plain in both unfertilised and fertilised condition (Fig. 5).
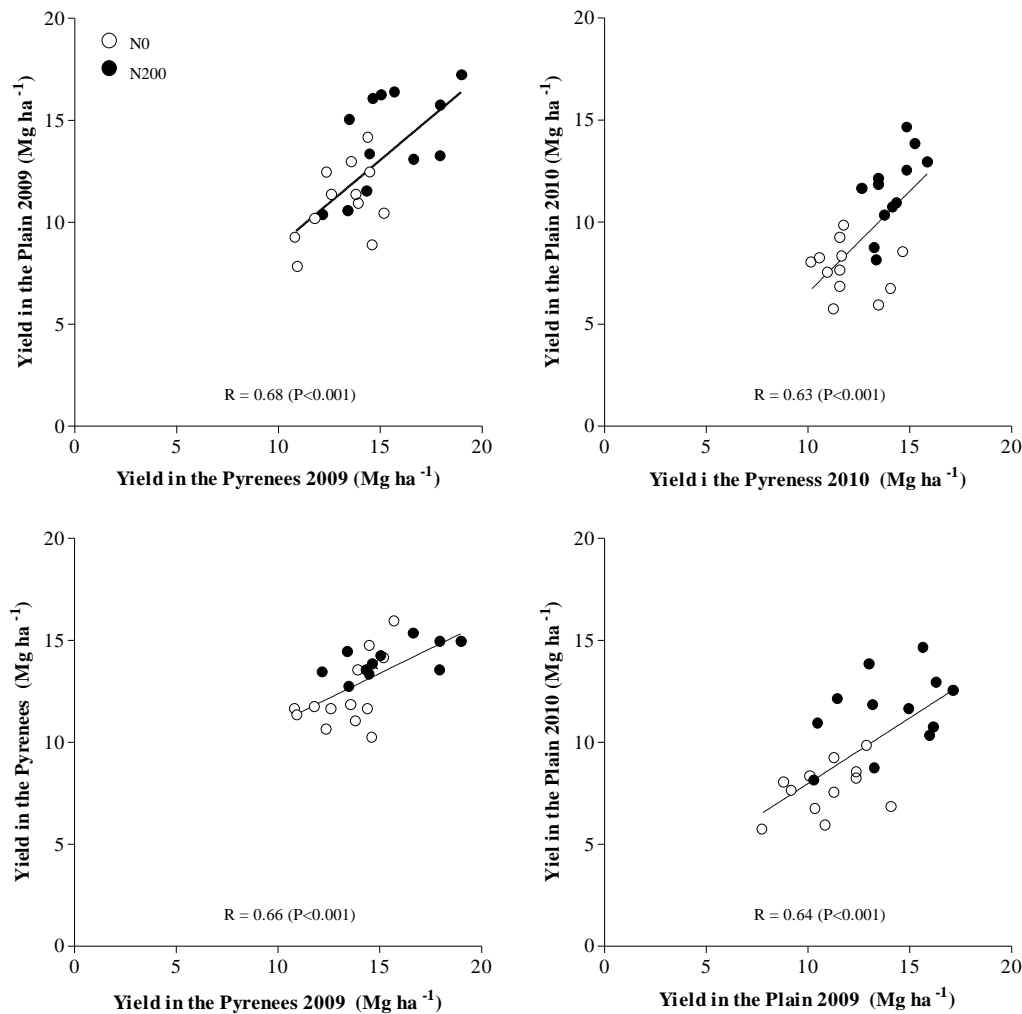
Figure 5. Grain yield relationship between unfertilised (open symbols) and fertilised (closed symbols) hybrids under different location x year combination: Plain of Lleida *vs* Pyrenees 2009 (upper left panel) and 2010 (upper right panel); Pyrenees 2010 *vs* 2009 (lower left panel) and Plana of Lleida (lower right panel).

A positive correlation between yield at the plain of Lleida and yield in the Pyrenees were found in 2009 (r = 0.68, P <0.001) and in 2010 (r = 0.63, P <0.001) (Fig. 5). Similarly, yield correlations between 2010 vs. 2009 in the plain of Lleida (r = 0.64, P <0.001) and in the Pyrenees (r=0.66, P <0.001) were significant. This indicated that yield ranking tended to maintain between both growing seasons and locations (Fig. 5, lower panels).

Grain yield was higher in the fertilised treatment, with higher N availability, than in the unfertilised control treatment (13.7±0.3 *vs* 11.0 ±0.4, respectively). The magnitude of responses was different in each location x year x hybrid combination (Figs. 4, 5).

In general, there was no significant relationship between grain yield and timing from sowing to silking in any treatment and location (Fig. 6). However, long-cycle hybrids tended to perform better than short-cycle hybrids, though short-cycle hybrids performed reasonably well under fertilized conditions (insets Fig. 6).

Then, (i) using hybrids of longer cycle in the Pyrenees might be an alternative to increase the production, if farmers are prepared to assume higher risks of frost damages, and (ii) if in the plain farmers envisage advantages of having more than one crop per season or advantages in releasing the field earlier, short-cycle hybrids would produce reasonably high yields.

2.3.3 Relationship between yield and yield determinants

Grain yield differences were linearly and closely related to grain number per m^2 and to a lesser extent to grain weight as well (Fig. 7). These relationships were clear and consistent in all the tested conditions (year x location x N).
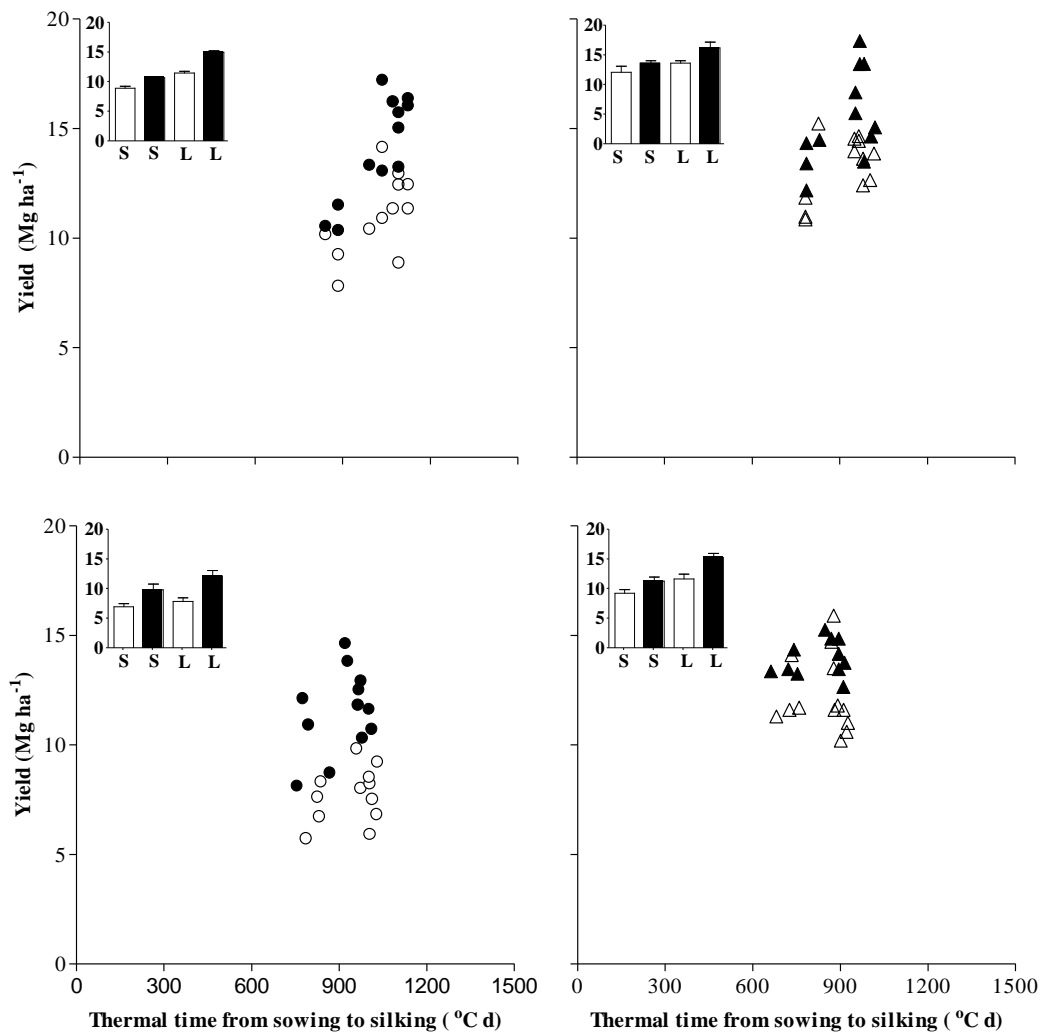
Figure 6. Relationship between grain yield and timing from sowing to silking (stigmas emission) for each experiment conducted at the plain of Lleida (left panels) and the Pyrenees (right panels) during 2009 (upper panels) and 2010 (lower panels). Open symbols unfertilized treatment and closed symbol fertilizer treatment (circles and triangles stand for the plain and the Pyrenees, respectively). The insert bar graphs show yields average obtained from short- (S) and long-cycle (L) cultivars. The segments represent the standard deviation of the means.

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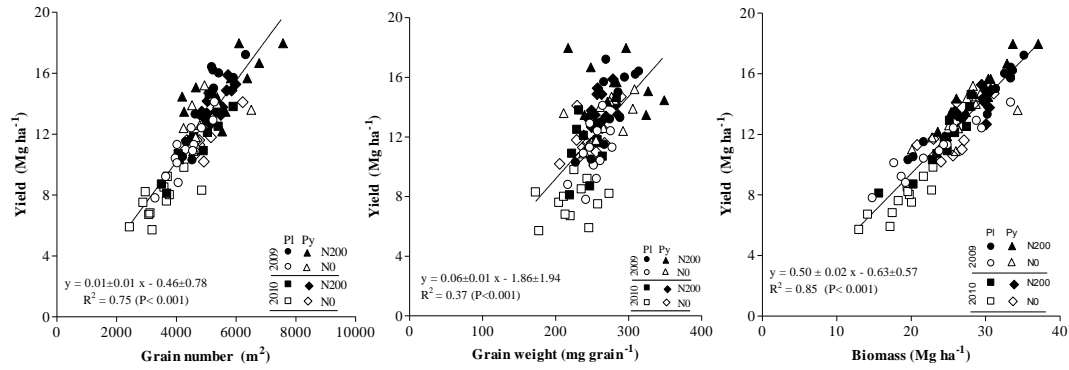
Figure 7. Relationship between grain yield and grain number per unit area (left panel) and grain weight (middle panel) and total biomass at maturity (right panel) for each experiment conducted at the plain of Lleida and the Pyrenees in 2009 and 2010. Open symbols correspond to unfertilised treatment and closed symbols to fertilised treatments (plain, circles and squares, 2009 and 2010 respectively; Pyrenees. triangles and rhombuses 2009 and 2010 respectively).

In addition, a linear and strong relationship was found between yield and total biomass at maturity for all treatments (Fig. 7).

Crop growth between flowering and maturity was an interesting aspect of the strong relationship between grain yield and biomass produced by the cultivar (Fig. 8). In our experiments, the relationship between grain yield and biomass accumulated during grain filling was in general stronger and more consistent than the relationship between yields and biomass at silking (Fig. 8).

Not only total biomass at flowering was not correlated with yields, but also leaf area index at silking did not explain yield differences (data not shown).
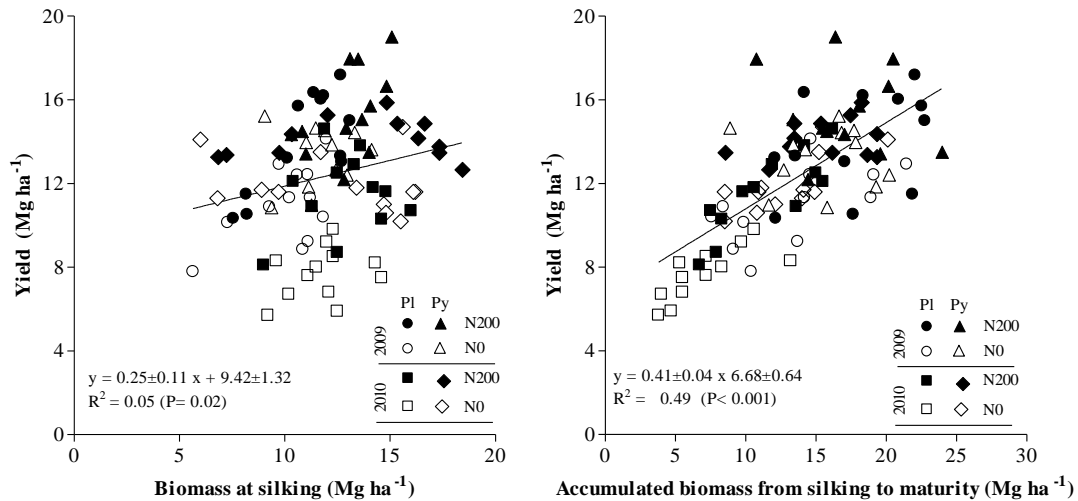
Figure 8. Relationship between grain yield vs aboveground biomass at silking stage (left panel) or accumulated biomass during grain filling (right panel) for each experiment conducted at the plain of Lleida and the Pyrenees during 2009 and 2010. Control unfertilised open symbols and closed symbols correspond to fertilised treatments. (plain of Lleida circles and squares, 2009 and 2010 respectively; Pyrenees triangles and rhombuses 2009 and 2010, respectively).

2.3.4 Overall considerations

Two principal components explained more than 60% of the G x E variability (Fig. 9). Overall the variation explored it is worthwhile highlighting that

(i)   Naturally, long-cycle hybrids clearly separated from short-cycled ones across the second component and Lapopi was the longest of the short-cycle hybrids and PR31N28 was mixed with the other long-cycle hybrids.

(ii)   Regarding yield, it was mainly captured by the first principal component, implying that the length of the cycle was in general not very relevantly determining yield (beyond the fact that collectively long-cycle hybrids tended to outyield short –cycle hybrids, but variations within these groups were irrelevant). In the context of this first component both Lapopi and PR31N28 were reasonably representing the relatively short- and the relatively long-cycle hybrids.

(iii)   Yield was more strongly associated with biomass than with harvest index, and with grain number than with the average weight of the grains. Plant height varied little and that variation was largely irrelevant for yield determination.

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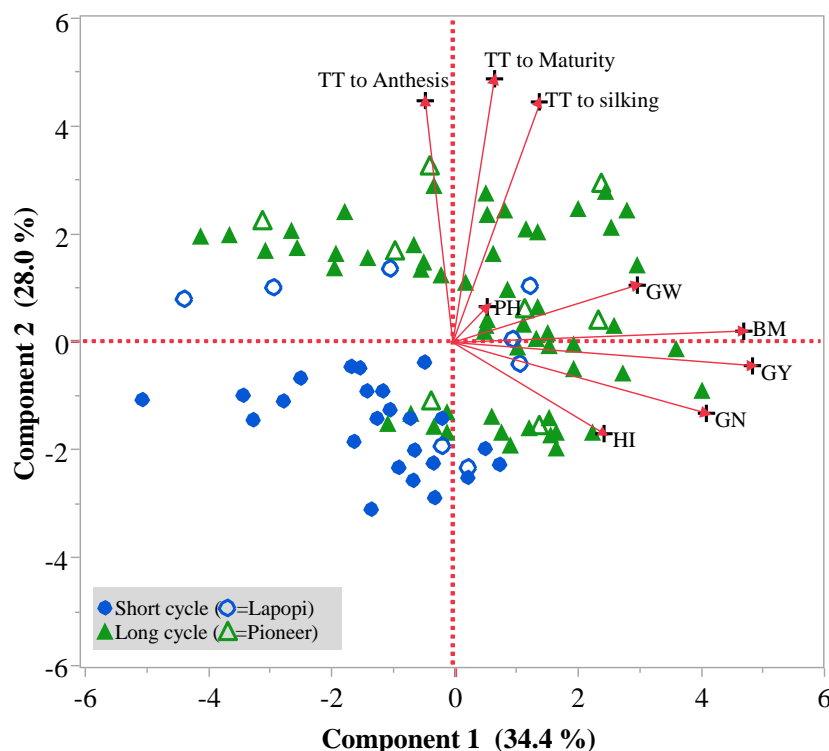
Figure 9. Biplot of the 1st and 2nd principal components for all hybrids in the 8 environments. Variables are represent by letters, hybrids x environments by vectors, and long- and short-cycle hybrids by triangles and circles, respectively (open triangles and circles stand for PRN31N28 and Lapopi, respectively). GY= Grain yield, BM= Biomass, GN= Grain number, GW= Grain weight, HI= Harvest Index, TT= Thermal time from sowing, PH= Plant height.

2.4 Discussion

2.4.1 Crop phenology and yield

Unfortunately no continuous gradient in the cycle length was achieved by the selection of hybrids FAO 400, 450, 600, 650, 700 and 750. Discrete variation in the cycle duration was achieved from sowing to silking in any tested environment. However, it was possible to at least achieve a bi-modal representation of 4 hybrids with relatively short duration to silking and 8 hybrids with relatively long cycle, which was consistent across the environments explored (Fig. 3). Other studies (Braga et al., 2008; Malik, 2014) also found almost no differences in silking and maturity date for hybrids classified as FAO 500, 600 and 700. This consistent lack of consistency of the FAO classification requires a revision and the identification of a mare consistent approach to classify hybrids in order to establish phenological differences.

An interesting observation was that phenological differences in the cycles to silking were independent to the duration of grain filling period, which gives an indication that the duration of the vegetative and reproductive stages are independent (Slafer et al., 2009). This in turn may open room to the design of ideotypes with similar cycle to maturity but different partitioning of that time between pre- and post-silking durations.

N fertilization does not consistently affect crop development and dates of silking and physiological maturity were virtually the same in contrasting conditions of availability of N. These results confirm previous evidence showing that fertilization affected little (Massignam et al., 2009) or not affected (Hall et al., 2014) the duration of the crop cycle. Therefore, comprehensive studies of maize adaptation to different availability of N do not consider silking date (or the overall cycle) as an important trait for understanding responsiveness to N (*e.g.* Cirilo et al., 2009).

2.4.2. Yield and its determinants

In general, yield potential was greater in 2009 than in 2010. This difference between years was probably due to the photothermal quotient around silking stage, as in maize, grain yield potential is related to a higher photothermal quotient around silking (Andrade et al., 2000). During July 2009, slightly higher radiation and lower temperatures than in July 2010, may be responsible for a higher yield during the first year. Moreover, this could be the reason why yields at the Pyrenees tended to be higher than at the plain of Lleida.

Among all hybrids, grain yield was positively associated with final grain number per unit area more than with grain weight, as found by other authors (Otegui and Banhomme, 1998), Andrade et al., 2000, Capristo et al., 2007). In adition, grain yield was related to total biomass at maturity but unrelated to total biomass at silking, revealing a strong relationship with accumulated biomass from silking to maturity (Fig. 4).

The differences between hybrid performances were independent of differences in cycle length but if the comparison is restricted to the average of all short- and long-cycle hybrids, it can be confirmed that the shorter-cycle hybrids had lower production than the long-cycle hybrids. Even though, grain yield for the short-cycle hybrids were reasonably high. A valuable aspect is that there was a tendency for higher yielding

hybrids in a condition (location per year) tended to be the highest performance in another location or in another year. The tendency for higher yield potential genotypes to present better overall performance under lower yielding conditions have been shown in the literature (Evans and Fischer, 1999; Abeledo et al 2003;. Slafer and Araus, 2007).

As long-cycle hybrids tended to yield more than short-cycle hybrids even at the Pyrenees, they result in an alternative to potentially higher production in this region, should the farmers be prepared to assume higher risk (although in the two growing seasons temperatures were very low, it is difficult to discard that lower temperatures may occur and may affect grain growth more markedly). On the other hand, at the plain, it would be possible to sow short-cycle hybrids as they proved to have reasonably high yield potential if other productive scenarios are planned with as a rotation with two crops per year.

Overall the variations analysed (two locations, two years, and two levels of N fertilisation) Lapopi and PR31N28 represented reasonably well the relatively short- and the relatively long-cycle hybrids. This provides support to have used them for realistic conclusions in a wider context in the more detailed studies reported in Chapter III.

2.5 Acknowledgements

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Chapter III

Responsiveness of senescence traits and yield components to nitrogen fertilisation in long and short cycle maize hybrids grown under a warm and a cool location

3.1. Introduction

Climate model projections suggest that higher temperatures will become commonplace in most regions where grain crops are produced. These deleterious effects of high temperature on crop yields are well documented in the literature. A frequently assigned major impact of high temperature is the acceleration of leaf senescence (Wahid et al., 2007) which might be responsible for the reductions in grain weight and yield. Therefore, it is critical identifying genetic and management tools to mitigate the effect of high temperatures on yield. At low latitudes, tememprature and radiation do not vary much along the year and long-season híbrids are generally the most situable because they use more of the availiable resource than Shorter híbrids (see Chapter II, Capristo et al., 2007). But, at high latitudes, radiation and temperature decrease markedly during grain filling and grain yield maybe penalised in longer cycle hybrids (Chang, 1981; Muchow, 1990; Ruget, 1993). This pattern is emphasised with the altitude of the location.

Nitrogen (N) fertilisation is one of the most widely applied management practices in grain crops worldwide. In many regions, crops are frequently well fertilised to maximise productivity. However, there have been limited efforts to elucidate to what degree hybrids of different crop cycle and altitude (thermal characteristics) of the location may interact with N responsiveness in determining leaf senescence during grain filling and yield. The identification of easily measured physiological traits contributing to yield under specific environmental conditions may perhaps contribute to genotypic selection for maize, especially in adverse environments. As is well known, maize phenology is clearly affected by temperature stress, even though maize crop is cultivated at latitudes ranging from the equator to 50° (North and South), at altitudes ranging from sea level to 3000 m elevation, under temperature ranging from cool to very hot. As far as we are aware, no studies have reported on the interaction between nitrogen fertilisation and cycle duration with some additive effect generated for differential altitude. Quantifying this interaction would be instrumental in breeding (contributing to the definition of ideotypes) and management (contributing to adjust needs of fertilisation depending on crop cycle and thermal conditions of crop growth).

Recent studies have shown that some traits were responsible for genetic gains estimated for maize grain yield, and the expression of these characters was dependent with the growing conditions (Luque et al., 2006). In this context, several traits have been

associated with maize yield depending on the environmental conditions, such as grain number (Andrade et al., 1999; Echarte et al., 2004) and grain weight (Borrás and Otegui, 2001) under optimal field conditions, stomata conductance under drought stress (Cairns et al., 2012; Witt et al., 2012), maximum rate of photosynthesis under heat stress (Crafts-Brandner and Salvucci, 2002), isotopic composition of C and O (Cabrera-Bosquet et al., 2009a,b), and reduced leaf senescence sustaining leaf photosynthesis during grain filling (Tollenaar and Lee, 2006). Differences in yield were attributed to the effects of N stress on grain number (Gallais and Hirel, 2004; Worku et al., 2007). Furthermore, sub-optimal N supply may decreased leaf area duration (Wolfe et al., 1988), and photosynthetic rate per unit leaf area (Muchow and Sinclair, 1994) resulting in a clear reduction of the total photosynthetic capacity of the plant expressed as leaves senescence. More specific, leaf senescence in maize due to N stress differed from the physiological senescence of a senescent hybrid (Pommel et al., 2006). The capacity to uptake N in post-silking can be related to leaf senescence, by increasing leaf longevity, thus prolonging the capacity of the plant to absorb mineral N (Moll et al., 1987). This was only found in modern hybrids with the highest yields (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999; Tollenaar, 1991). However, it was demonstrated that yield could be diminished with the sensitivity to environmental changes during grain filling (Paponov et al., 2005).

In addition, to maintain high yields, the length of the growing cycle is one of the most important traits determining hybrid adaptability to the environment. Increase the cycle duration become favourable to the plant for sink production (Capristo et al., 2007). For instance, low yields in short cycles were due to the limitation in sink production during grain filling (Dwyer et al., 1994; Otegui et al., 1996).

As highlighted in Chapter II, in the province of Lleida (Catalonia, NE Spain) maize is sown from the region of the plain to the valleys of the Pyrenees, along an altitudinal gradient. It was shown that differences in yield among hybrids were not strongly related to cycle duration, and that a short-cycle cultivar may yield reasonably well in the plain while long-cycle hybrids may offer promising alternatives in the Pyrenees (Chapter II). In the present chapter, a more detail study in a short- and a long-cycle hybrid further analysing the interactions between nitrogen (N) availability and contrasting altitudes. As farmers usually fertilise maize with high doses (*c*. 300 KgN ha^{-1}) and the region is facing serious pollution problems due to excessive fertilisation (Berenguer et al., 2009;

Cela et al., 2011). The main objective of the study was to determine differences between hybrids of short and long cycle at locations of contrasting altitude and under low and high N availabilities in terms of yield components, leaf senescence and N-economy traits.

3.2 Materials and methods

3.2.1 Treatments and trial sites

Four field experiments were carried out to compare the performance of a short- and a long-season hybrid under contrasting N availabilities. The experiments were conducted at two locations differing in altitude being one then representative of relatively warm environments and the other of relatively cool environments of maize production in Catalonia (NE Spain). The warm environment was a location in the plain of the province of Lleida close to Algerri (41º47´41´´ N; 0º38´52´´ E, 230 m alt) and the cool environment was a field in a Valley in the middle of the Pyrenees close to La Seu d`Urgell (42º 20' 40.6" N; 1º 25' 47.4" E; 730 m alt), in both locations experiments were carried out during two consecutive experimental years, 2009 and 2010 (Table 1).

Treatments consisted in a comparison of two maize hybrids differing in their growing cycle duration combined with two different N availabilities (unfertilised and fertilised with 200 kgN ha^{-1} applied as urea at V6, Ritchie and Hanway, 1982). These hybrids were chosen presuming (based on information from networks and personal communications with advisors and farmers) they do represent well modern, high yielding hybrids adapted to the region differing in cycle, Lapopi and Pioneer PR31N28 (classified as FAO 450 and FAO 700, respectively). In a parallel study we satisfactorily proven this presumption (Chapter II).

Table 1. Main soil properties before sowing in each location.

| Soil properties | Location | | | |
| | 2009 | | 2010 | |
	Low$_{Alt}$	High$_{Alt}$	Low$_{Alt}$	High$_{Alt}$
Sowing date	12-May	11-May	17-May	20-May
Sowing rate (plants ha^{-1})	80,025	85,000	84,030	85,000
N-NO3- (kgN ha^{-1})	175¥	150‡	141¥	129‡
Phosphorus Olsen (kgN ha^{-1})*	70	88.2	35.1	70
pH (Ext. 1:2:5 H2O)*	8.2	8	8.2	8
EC 25ºC (Ext.1:2.5 H20) dS/m*	0.4	0.1	0.2	0.1
Organic Matter (Walkley-Black, %)*	1.9	1.4	1.5	1.4
USDA textural soil classificatio)	Clay loam	Sandy loam	Clay loam	Sandy loam

Soil samples taken from *0-30 cm, ‡0-75 cm, ¥0-100 cm depth

Treatments were arranged in a split-plot design with genotypes as main plots (randomized in 3 blocks) and N availability as sub-plots. Experimental units (sub-plots) were 20 x 6 m^2 and 10 x 3m^2 in the plain and the Pyrenees, respectively. In both cases, three replicates were used for each experimental unit.

The trials were irrigated to maintain the crop free of water stress during the whole growth cycle. Insects and weeds were prevented by spraying recommended insecticides and herbicides at the doses suggested by their manufacturers.

Global radiation, minimum and maximum temperature, and precipitation were recorded daily at standard meteorological stations of the Agro-meteorological network of Catalonia, Spain, located close to the experimental fields. Mean and maximum temperatures during the growing season were higher in the Plain than in the Pyrenees in both years (Table 2). Also, global radiation was higher in the Plain than in the Pyrenees (Table 2).

3.2.2 Sampling, measurements and data analysis

Female flowering (silking) date were recorded when 50% of the plants in each experimental unit exhibit the silks. The duration of pre-and post-silking development was expressed in degree days, with a base temperature of 8 ^{o}C (Cirilo and Andrade, 1996).

Plant samples were taken for each experimental unit site. Dry weight and N percentage (Kjeldahl) was determined for different plant tissues obtained at silking (stem, leaf blades and small ear) and at physiology maturity (stem, leaf blades and grain). Grains were separated and weighted and counted to determine final grain number and average grain weight. N content (KgN ha^{-1}) was determined for each organ. Then N uptake was computed as the sum of total N content from each plant tissue. N utilisation efficiency was calculated as the ratio of grain yield (kg) and total of N uptake at maturity and finally N harvest index was calculated as the ratio of N content in the grains and the total N uptake expressed as a percentage.

Table 2. Temperature and global radiation during developmental phases for the two cycle hybrids and the two locations during 2009 and 2010.

Average temperatures (°C) during different developmental phases for two hybrids						
Season	Sowing to Silking		Silking to Maturity		Sowing to Maturity	
	Lapopi	PR31N28	Lapopi	PR31N28	Lapopi	PR31N28
Low$_{Alt}$ 2009	25.6	22.8	24.7	22.8	23.6	22.8
Low$_{Alt}$ 2010	21.8	22.2	23	21.7	22.4	21.9
High$_{Alt}$ 2009	19.9	19.9	20.3	17.95	19.7	18.9
High$_{Alt}$ 2010	18.5	19.2	18.8	17	18.8	18.1
Average maximum temperatures (°C) during different developmental phases for two hybrids						
Season	Sowing to Silking		Silking to Maturity		Sowing to Maturity	
	Lapopi	PR31N28	Lapopi	PR31N28	Lapopi	PR31N28
Low$_{Alt}$ 2009	29.7	29.9	32.1	30.2	30.8	30
Low$_{Alt}$ 2010	29.2	29.4	29.6	28.3	29.4	28.8
High$_{Alt}$ 2009	26.6	27.2	27.4	25.2	26.9	26.2
High$_{Alt}$ 2010	25.8	26.2	25.9	23.7	25.8	25
Average global radiation (MJ d^{-1}) during different developmental phases for two hybrids						
Season	Sowing to Silking		Silking to Maturity		Sowing to Maturity	
	Lapopi	PR31N28	Lapopi	PR31N28	Lapopi	PR31N28
Low$_{Alt}$ 2009	26.5	26.5	25.1	22.8	25.8	27.7
Low$_{Alt}$ 2010	27.5	27.4	23.4	22.2	25.3	27.7
High$_{Alt}$ 2009	24.5	24.7	20.5	17.5	22.4	23.3
High$_{Alt}$ 2010	24.5	24.6	20.8	17.8	22.6	21.5

In each experiment chlorophyll content was indirectly assessed with a SPAD meter (chlorophyll meter SPAD 502, Minolta Co. Ltd., Japan). Chlorophyll dynamics were measured weekly from silking to maturityin the ear leaf (all experiments) and in 4

leaves (only in 2010 experiments). A bi-linear relationship between chlorophyll content in the ear leaf or in the 4 leaves and thermal time after silking in all the treatments and experiments was established. The parameters were the chlorophyll content at silking (intercept), the onset of chlorophyll loss (the timing after silking when the loss of SPAD units started irreversibly), and the rate of chlorophyll loss from then to maturity.

All data were analyzed by ANOVA to evaluate the effects of treatments and their interactions. A t-test was used to determine significant differences ($P < 0.05$; <0.01; <0.001) between means. The relationship between variables was analyzed by linear regression. A multivariate analysis of principal components was performed to the main variables and for all treatments (2 hybrids, 2 locations, 2 years and 2 N availabilities).

3.3 Results

3.3.1 Weather conditions and crop phenology

Increases in cycle length did not resulte in major changes in the average temperature from emergence to silking period and from silking to maturity (Table 2). Average temperature during vegetative growth varied from c.18 to 25ºC according to the location. But temperatures were in average of the whole growing season lower in the Pyrenees (18.8 ºC) compared that at the low altitude location (22.9 ºC; Table 2). There were no differences in average daily incident radiation from emergence to silking between the two hybrids (25.7 MJ d^{-1}), but the long-cycle hybrid had lower levels of incident radiation from silking to maturity (20.1 MJ d^{-1}).

As expected, hybrids differed in the duration from sowing to silking ($P< 0.001$ Fig. 1). Averaged across the four experiments, Lapopi (the FAO 450 hybrid) was c.160 ºCd shorter than PR31N28 (the FAO 700 hybrid). However, the duration between silking to maturity was similar between the two hybrids in the plain, while in the Pyrenees the duration from silking to maturity was c. 100 ºC d shorter in PR31N28 than in Lapopi presumably because of frost occurring slightly before maturity of the longer hybrid (Fig. 1). This frost affected only the very last part of the grain filling period when grain weight was already virtually maximised. Nitrogen availability had no effect in 6 out of the 8 environments in cycle duration (Fig. 1).
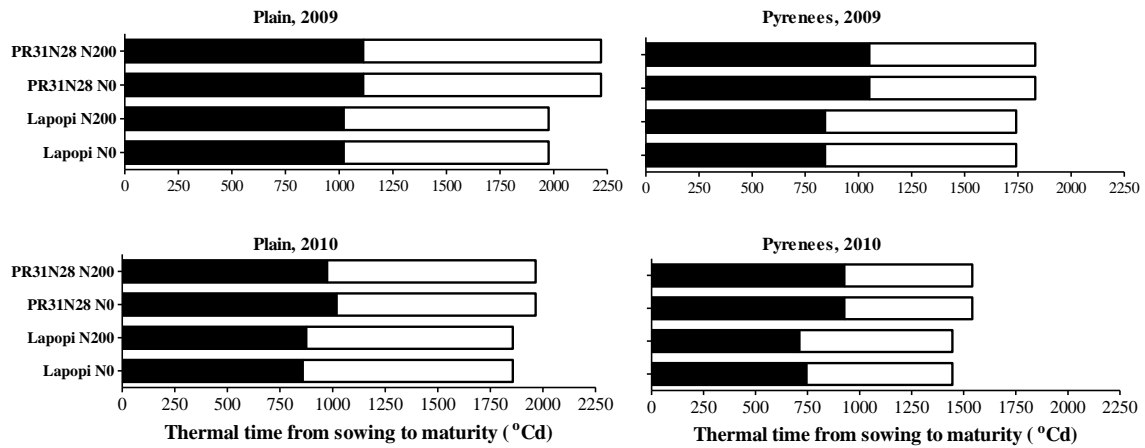
Figure 1. Thermal time accumulated during the whole growing cycle for the two hybrids (Lapopi, FAO 450 and PR31N28 FAO 750) in the two locations (low and high altitude). Closed bars represent the duration from sowing to silking and open bars from silking to maturity. Left panels are from the Plain and the right panel from the Pyrenees.

3.3.2 Grain yield and it components

Considering all experiments and treatments, yield varied from *c.* 8.6 to 18 Mg ha^{-1}, being in average higher during the first than the second growing seasons (Table 3 top part; Fig. 2). As expected, yield was higher under fertilised than unfertilised conditions, 14.5 and 11.6 Mg ha^{-1}, respectively. In addition, grain yield was higher in the Pyrenees than in the plain 14.3 *vs* 11.9 Mg ha^{-1}, respectively. But, averaging overall conditions, yields for PR31N28 and Lapopi cycle were similar, 13.1 *vs* 13.0 Mg ha^{-1}, respectively (Table 3 top part; Fig. 2). However, when we compared the relative response to nitrogen fertilisation in each cycle hybrid, the responsiveness to N fertilisation was clearly higher in PR31N28 than in Lapopi (averaging across experiments, 30 *vs* 9%, respectively, Table 3 top part). Also, the response was in average higher in the low altitude than in the high altitude location, being respectively, 23 *vs* 18 % (Table 3a).

The major effects on yield were produced by the main factors year and N availabilities and, to a lower degree, the location while the genotypic effects were much smaller and mainly not significant (Table 3, bottom part), but interestingly the interactions between location x genotype and N x genotype were also statistically significant (Table 3, bottom part). The effects of different sources of variation on yield, was also presented on some of its determinants: grain number, grain weight and total biomass at maturity (Table 3, bottom part).

Total biomass at silking and LAI at silking were mainly affected by the genotype and N availabilities with no interaction with the location or experimental year (Table 3, bottom part), indicating that in the context of the sources of variation considered, growth after silking has been far more relevant than growth to silking for the determination of yield, which might be a major reason for the lack of hybrid consistent differences in yield despite their consistent differences in time to silking.

Table 3. Yield, grain number, grain weight, biomass at silking and at maturity and leaf area index (top part) and mean square values of the ANOVA (bottom part) for the two maturity type hybrids and the two locations during 2009 and 2010. The relative response to the unfertilised control is shown (N resp).

Year	Altitude	Hyb	Nit	GY (Mg ha⁻¹)	N resp (%)	GN (m²)	N resp (%)	GW (mg grain⁻¹)	N resp (%)	BM (Mg ha⁻¹)	N resp (%)	BS (Mg ha⁻¹)	N resp (%)	BS-M (Mg ha⁻¹)	N resp (%)	LAI Silking	N resp (%)
2009	Low	Lapopi	N0	11.8	8.1	3828.4	8.2	305.9	0.3	25.0	2.8	9.0	4.2	16.0	2.0	3.4	8.6
			N200	12.7		4141.6		306.8		25.7		9.4		16.3		3.6	
		PR31N28	N0	13.2	42.4	4562.2	21.5	287.5	16.6	28.1	32.9	12.4	29.3	15.7	35.7	4.3	17.7
			N200	18.8		5544.0		335.3		37.3		16.1		21.3		5.0	
	High	Lapopi	N0	16.6	7.7	5114.4	7.2	327.5	-0.5	32.2	6.1	12.4	-11.0	19.8	16.8	4.2	0.2
			N200	17.9		5482.5		325.8		34.1		11.0		23.1		4.2	
		PR31N28	N0	12.9	28.2	4340.3	13.5	295.2	13.7	27.9	21.0	12.2	7.5	15.7	31.5	5.8	2.1
			N200	16.5		4925.1		335.5		33.7		13.1		20.6		5.9	
2010	Low	Lapopi	N0	8.6	13.8	4391.5	2.2	193.5	9.0	17.3	18.1	8.1	45.7	9.2	-6.5	3.5	44.7
			N200	9.8		4487.7		210.9		20.4		11.9		8.6		5.1	
		PR31N28	N0	7.2	75.1	3211.3	37.6	235.3	21.9	17.3	55.0	11.7	20.9	5.6	125.4	4.8	10.1
			N200	12.7		4419.4		287.0		26.8		14.1		12.7		5.3	
	High	Lapopi	N0	13.0	9.3	4812.6	-0.9	267.7	11.8	25.5	11.4	11.1	11.2	14.4	11.6	3.5	4.2
			N200	14.2		4768.2		299.3		28.4		12.4		16.0		3.7	
		PR31N28	N0	10.0	34.3	4199.5	17.3	239.1	15.0	24.4	25.5	13.8	0.4	10.5	58.5	6.4	15.0
			N200	13.5		4924.2		274.9		30.6		13.9		16.7		7.3	

Mean square values of ANOVA

Source of variation	DF	GY (Mg ha⁻¹)	GN (m²)	GW (mg grain⁻¹)	BM (Mg ha⁻¹)	BS (Mg ha⁻¹)	BS-M (Mg ha⁻¹)	LAI Silking
Year	1	183.9**	1391490.1ns	49133.0***	534.5**	0.4ns	562.4***	2.0ns
Location	1	72.1*	2971120.0*	7718.6*	280.5*	9.7ns	185.9*	6.6*
Year*Location	1	4.8ns	31304.6ns	2097.9ns	43.7ns	2.6ns	25.0ns	0.4ns
Block (Year*Location)	8	9.5*	467432.3ns	968.5*	32.3*	3.5ns	20.2ns	0.9ns
Genotype	1	0.04ns	152110.5ns	509.8ns	56.8ns	90.1**	3.8ns	34.5***
Year*Genotype	1	3.9ns	1181735.0ns	1127.4ns	1.2ns	0.7ns	0.1ns	1.0ns
Location*Genotype	1	59.1**	1343044.7ns	7788.3**	115.1*	17.7ns	42.5*	6.6**
Year*Location*Genotype	1	10.5ns	3401340.2*	3583.0*	36.2ns	8.2ns	9.9ns	2.9*
Genotype*Block (Year*Location)	8	4.0ns	311132.0ns	418.2ns	13.3ns	4.7ns	6.4ns	0.4ns
N	1	97.1***	3358962.0**	9399.3***	293.0***	22.8*	152.2**	3.5*
Year*N	1	0.007ns	12982.3ns	451.4ns	3.0ns	2.8ns	0.003ns	0.7ns
Location*N	1	2.6ns	174979.7ns	25.9ns	6.0ns	16.3*	2.6ns	0.71ns
Year*Location*N	1	0.02ns	14899.9ns	13.6ns	0.4ns	0.03ns	0.2ns	0.0001ns
Genotype*N	1	34.2**	1434945.4*	3037.3**	91.8**	1.8ns	68.2*	0.02ns
Year*Genotype*N	1	0.1ns	185868.2ns	477.8ns	1.4ns	12.1ns	5.2ns	0.1ns
Location*Genotype*N	1	3.5ns	118438.3ns	227.7ns	11.3ns	0.2ns	8.8ns	0.4ns
Year*Location*Genotype*N	1	0.02ns	2224.8ns	119.0ns	0.4ns	0.2ns	0.04ns	0.9ns

Abreviations, GY=grain yield; GN= grain number; GW= grain weight; BM=Biomass at maturity; BS= Biomass at silking; BS-M=Biomass from silking to maturity; LAI= leaf area Index
Significant at the probability level of p *<0.05,**<0.01,***<0.001; ns:not significant

Pooling all data together grain number and grain weight were both significantly and highly correlated with grain yield (Fig. 2).
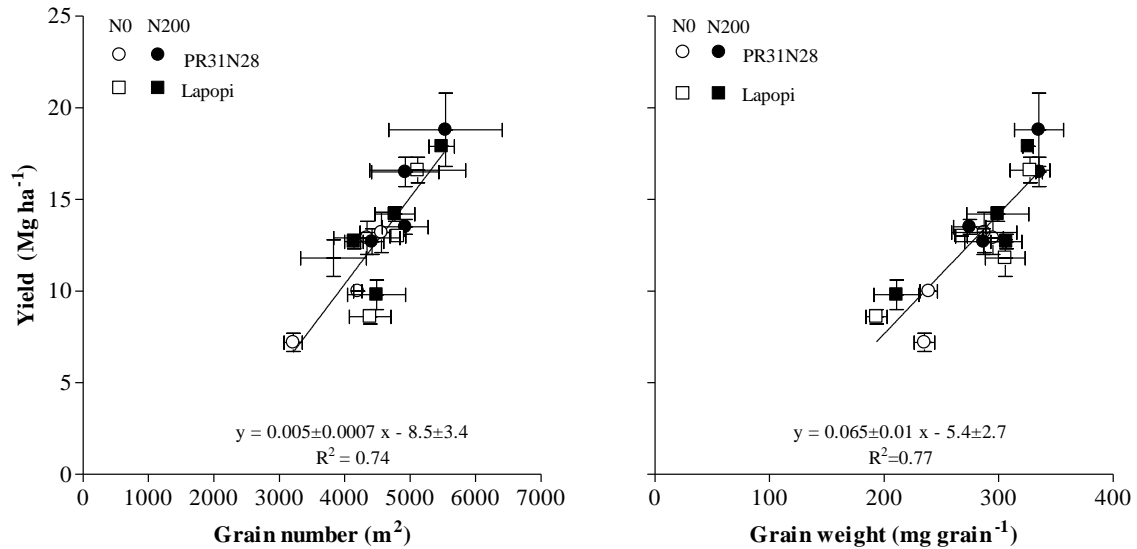
Figure 2. Relationship between yield *vs* number grain a (left panel), and grain weight (right panel). Open symbols represent unfertilised treatment and closed symbols fertilised. Square symbols represent Lapopi and circles PR31N28. Each data point corresponds to one single doses of N under determinate combination of genotype and location.

Although both components explained similar proportions of yield variation across years, locations, N availabilities and hybrids, it cannot be stated that both were equally relevant: the range of variation in grain size was smaller than that of grain number (Fig. 2).

3.3.3 SPAD values during grain filling

As expected SPAD values measured during grain filling in the ear leaf, were in general higher under N200 than under N0 treatments (Fig. 3), though in some cases there were no clear differences.
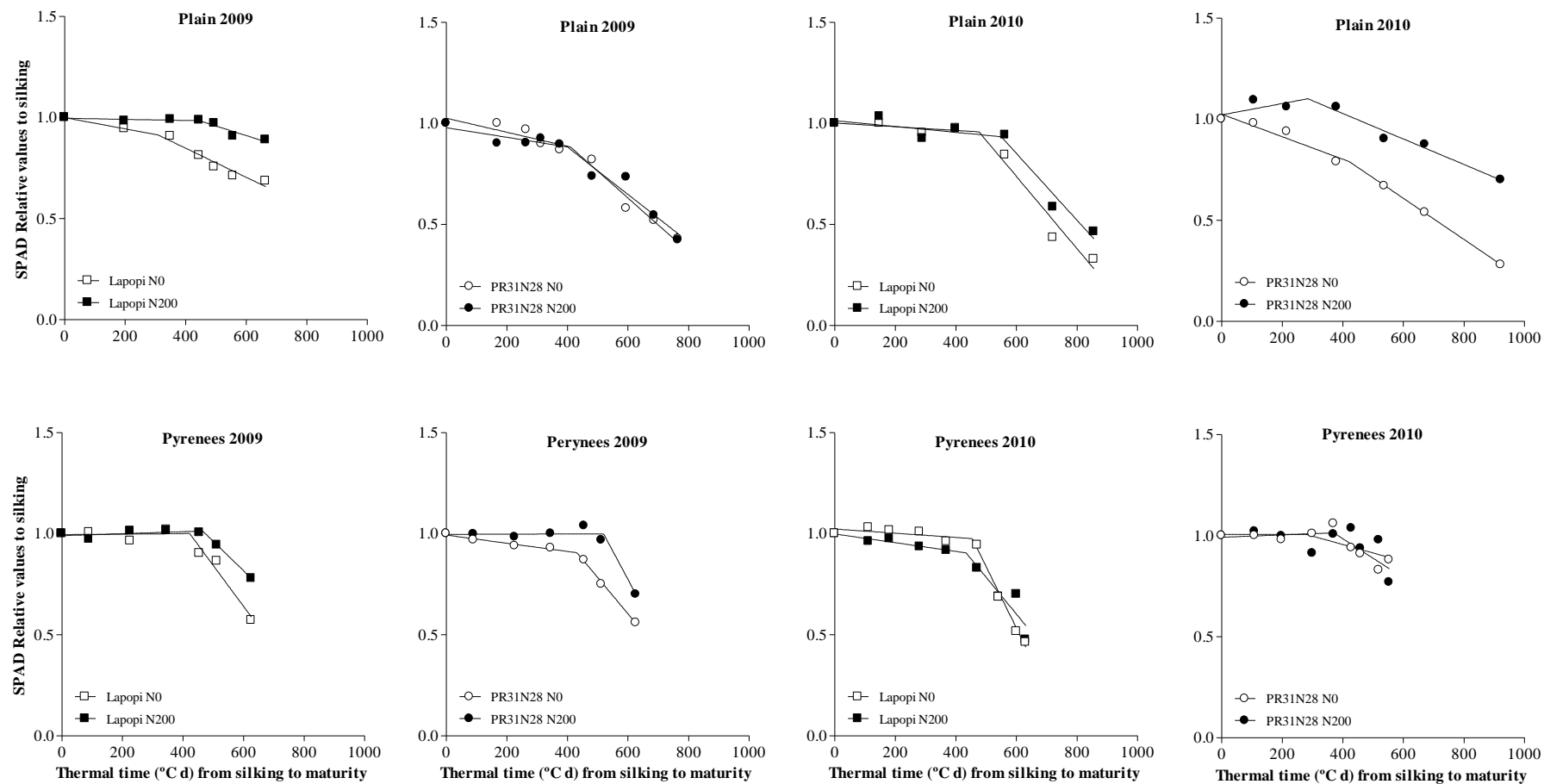
Figure 3. Chlorophyll leaf contain dynamic for the ear collar leaf from silking to maturity stage. Each data point represents the relative values respect to the value measured at silking stage. Open symbols represent N0 and closed symbols N200 treatments. Square symbols correspond to Lapopi and circles to PR31N28.

Therefore, in general the area under the curve (integrating different parameters of chlorophyll loss during grain filling) responded positively to N fertilisation (Table 4). Overall the AUC was c. 34,000 SPAD units (ºC d)$^{-1}$ under N200 and c. 29,000 SPAD units (ºC d)$^{-1}$ under N0 (Table 4), but no significant differences were consistently found between the two hybrids (averaging across conditions c. 33,000 and 30,000 SPAD units (ºC d)$^{-1}$ for the FAO 750 and 450 hybrids, respectively; Table 4); although differences in the relative response to N fertlisation in AUC was in all cases higher in the long-than in the short-cycle hybrid (Table 4), consistently with the higher responsiveness of yield in PR31N28 than in Lapopi (see above).

Table 4. Bi-linear relationship between chlorophyll content in the ear leaf or 4 leaves indirectly assessed through SPAD measurements, and thermal time after silking in all the treatments and experiments. The parameters shown are the chlorophyll content at silking (intercept), the onset of chlorophyll loss (the timing after silking when the loss of SPAD units started irreversibly), and the rate of chlorophyll loss from then to maturity. The coefficient of determination for each regression is given as well as the area under the curve of chlorophyll content (AUC) over thermal time as an integrated assessment of leaf photosynthetic capacity throughout grain filling. AUC correspond to leaf area duration SPAD units per thermal time (ºC d). All data point used in this analysis corresponds to a chlorophyll level in a determinate moment of the grain filling. The relative response to the AUC unfertilised treatment is shown.

Year	Altitude	Hybrid	Nitrogen	Parameters of the bilinear model				Area under the curve	
				Chlorophyll at silking (SPAD units)	Onset of chorophyll loss (ºC d)	Rate of Chloropyll loss (SPAD units[ºC d]$^{-1}$)	R^2	(SPAD units x ºCd 10^3)	N response (%)
2009	Low	Lapopi	N0	59.4±1.4	227.7±63.0	-0.044 ± 0.008	0.95***	35.1	11.6
			N200	60.3±0.5	430.6±30.9	-0.029 ± 0.006	0.92***	39.2	
		PR31N28	N0	46.9±1.0	314.9±46.6	-0.059 ± 0.007	0.97**	30.1	26.0
			N200	57.6±1.6	349.5±45.5	-0.071 ± 0.01	0.95***	38.0	
	High	Lapopi	N0	57.5±1.2	467.6±28.0	-0.15 ± 0.034	0.94**	34.1	5.9
			N200	59.3±0.5	473.3±17.1	-0.086 ± 0.012	0.97***	36.1	
		PR31N28	N0	46.3±1.1	343.6±30.2	-0.064 ± 0.01	0.96**	26.3	14.6
			N200	49.8±0.5	497±11.0	-0.117 ± 0.013	0.98***	30.1	
2010	Low	Lapopi	N0	41.0±2.0	390.1±58.3	-0.062 ± 0.01	0.95**	28.1	32.6
			N200	49.3±1.4	522.4±40.4	-0.083 ± 0.103	0.96**	37.3	
		PR31N28	N0	34.5±0.5	167.6±22.2	-0.032 ± 0.001	0.99***	22.6	58.2
			N200	42.3±1.7	379.1±165.6	-0.026 ± 0.01	0.81*	35.7	
	High	Lapopi	N0	55.8±0.8	446.8±11.3	-0.17 ± 0.029	0.99***	32.2	9.4
			N200	60.8±1.6	405±44.8	-0.116 ± 0.029	0.92***	35.2	
		PR31N28	N0	42.5±0.7	370±49.8	-0.039 ± 0.133	0.83**	22.8	28.5
			N200	54.5±2.5	279.4±196.3	-0.023 ± 0.02	0.31ns	29.4	

Significant at the probability level of p *<0.05,**<0.01,***<0.001; ns: not significant

No relationships between SPAD values or AUC with grain weight were found in any case (data not shown). In addition, no differences in the SPAD units and senescence pattern were found between the high and low latitude location (Table 4).

3.3.4 Nitrogen uptake and use efficiency

Total nitrogen uptake varied between c. 111.4 and 371.8 kgN ha^{-1} (Table 5, top part). In general, nitrogen uptake was higher under N200 (298 kgN ha^{-1}) than under N0 treatments (216 kgN ha^{-1}). There were no differences between the hybrids in N uptake at silking or maturity (Table 5), but the relative response to nitrogen uptake to fertilisation was clearly higher in the long cycle than in the shorter cycle hybrid, 40 *vs* 15 %, respectively (Table 5, top part).

There were positive relationships between total biomass at maturity (R^2= 0.94; p≤0.001) or yield (R^2=0.86; p≤0.001) and N uptake during the whole growth cycle for both hybrids in all experiments. The main effects on N uptake at maturity were related to the year and the N availability (Table 5, bottom part).

N utilisation efficiency tended to be higher in the unfertilised treatments; the range explored for this variable was from 40 to 60 kg$_{grain}$ kg$_N^{-1}$ in the combination years for locations (Table 5, above part). As general average (hybrid and N availability) there were no major differences in NUE between the locations, in the low altitude location 1000 kg extra of grains were obtained per each increase of 20 kg of N availability while that increase in yield would require additional 19.8 kg N available in the Pyrenees. In addition, genotypes differed in N utilization efficiency (Table 5, bottom part) and also in N content in leaves and grain (Table 5, bottom part), with a significant interaction genotype x N availability.

Table 5. Nitrogen uptake at silking and maturity, nitrogen content in the stems and grains (top part) and the mean square values of the ANOVA (bottom part) for the two cycle hybrids and the two locations during 2009 and 2010. The response relative to N0 is also shown (N resp).

Year	Altitude	Hybrid	Nitrogen	N uptk S (kg ha^{-1})	N resp (%)	N uptk M (kg ha^{-1})	N resp (%)	GNC (%)	N resp (%)	LNC (%)	N resp (%)	NUtE (kg$_{grain}$ kgN^{-1})	N resp (%)
2009	Low	Lapopi	N0	115.1	2.3	272.4	17.1	1.49	5.4	2.6	7.4	45.2	-9.8
			N200	117.7		319.0		1.57		2.8		40.8	
		PR31N28	N0	149.9	48.4	236.5	62.2	1.19	23.5	2.3	15.1	56.9	-11.8
			N200	222.5		383.5		1.47		2.6		50.1	
	High	Lapopi	N0	145.1	11.4	335.7	10.8	1.52	0.0	2.7	10.8	49.3	-2.1
			N200	161.7		371.8		1.51		3.0		48.3	
		PR31N28	N0	122.6	32.1	219.9	43.5	1.31	10.9	2.0	19.7	59.5	-8.1
			N200	161.9		315.5		1.45		2.4		54.7	
2010	Low	Lapopi	N0	58.5	105.5	140.6	16.1	1.19	-1.7	1.4	54.6	61.3	-0.3
			N200	120.2		163.3		1.17		2.2		61.2	
		PR31N28	N0	86.8	65.8	111.4	91.9	1.03	24.3	1.6	51.7	66.0	-9.9
			N200	143.9		213.8		1.28		2.4		59.4	
	High	Lapopi	N0	191.9	17.0	241.8	30.1	1.49	8.1	3.4	-7.8	53.7	-15.9
			N200	224.6		314.6		1.61		3.2		45.2	
		PR31N28	N0	132.34	59.5	171.2	77.6	1.19	29.4	1.9	52.3	58.9	-24.0
			N200	211.12		304.0		1.54		2.9		44.8	

Means square values of ANOVA

Source of Variation	DF	N uptk S (kg ha^{-1})	N uptk M (kg ha^{-1})	GNC (%)	LNC (%)	NUtE (kg$_{grain}$ kgN^{-1})
Year	1	135.3ns	118096.9**	0.2*	0.3*	393.9*
Location	1	21262.2**	35353.3ns	0.3*	2.4***	131.1ns
Year*Location	1	24921.3**	25781.4ns	0.2*	2.8***	768.9**
Block (Year*Location)	8	1312.8ns	7815.5ns	0.03ns	0.1ns	54.2ns
Genotype	1	1742.6ns	7768.0ns	0.2**	2.02**	382.9**
Year*Genotype	1	3575.3*	1312.0ns	0.01ns	0.04ns	169.6*
Location*Genotype	1	15448.9**	17236.6*	0.008ns	1.6**	1.3ns
Year*Location*Genotype	1	253.7ns	1809.7ns	0.04ns	0.2ns	7.7ns
Genotype*Block(Year*Location)	8	510.0ns	2732.0ns	0.01ns	0.1ns	20.2ns
N	1	24487.5***	80691.4***	0.3***	2.4***	402.4**
Year*N	1	1846.2ns	5.3ns	0.008ns	0.2*	28.8ns
Location*N	1	135.2ns	63.7ns	0.0002ns	0.1ns	21.2ns
Year*Location*N	1	25.9ns	3797.9ns	0.04ns	0.2ns	84.2ns
Genotype*N	1	3377.4ns	16812.3*	0.1*	0.4**	60.9ns
Year*Genotype*N	1	487.5ns	76.5ns	0.005ns	0.2ns	6.6ns
Location*Genotype*N	1	2.1ns	689.07ns	0.001ns	0.3*	0.1ns
Year*Location*Genotype*N	1	1806.8ns	84.5ns	0.0001ns	0.3*	0.9ns

N uptk S= Nitrogen uptake at silking, N uptk M= Nitrogen uptake at maturity; GNC= Grain nitrogen content, LNC= Leaf nitrogen content; NUtE= Nitrogen utilisation efficiency
Significant at the probability level of p *<0.05, **<0.01, ***<0.001, ns: not siginificant

3.3.5 Overall analysis with principal components

Two principal components explained more than 60% of the G x E variability (Fig. 4). Overall the variation explored it is worthwhile highlighting that

(i)   N fertilisation in general outyielded the N0 conditions, but not dramatically so nor always, indicating that in many circumstances it may not be necessary to fertilise (and this statement would be stronger if gross marging rather than yield would have been computed).

(ii)   Alike in the screening, simpler, analysis (Chapter II) the second component captured most of the variations due to hybrid length to silking and to maturity. As it was virtually unrelated to major variations in yield, yield was overall conditions not consistently different among the two hybrids (Fig. 4). In this more detailed approach it was clear that for this conditions fruiting efficiency (the number of grains set per unit of growth at silking) was negatively related to the cycle-length and the onset of senescence was positively related to the length of the cycle (the longer cycle hybrid tended to have lower efficiencies to set grains per unit growth but later onset of senescence), and none of these attribute were relevant to explain overall variability in yield (Fig. 4).

(iii)   Regarding yield, it was mainly captured by the first principal component, and was far more related to the capture of resources (biomass, N uptake) than to use of those resources (particularly yield was negatively related to N utilisation efficiency, which in turn was strongly negatively related to N uptake). Biomass accumulation during grain filling was more determinant of final biomass and yield than biomass differences at silking. Both yield components were responsible for the differences in yield across the whole range of conditions explored.

(iv)   Overall, there was a strongly negative relationship between grain protein percentage and N utilisation efficiency (Fig. 4), which is consistent with the concept that N accumulation in the grains is source-limited during grain filling and therefore the more yield is produced per unit of N uptake the more diluted the protein in the endosperm. This is also consistent with the positive relationship of grain protein percentage and the relative content of N in stems and leaves at silking (Fig. 4), which affected positively yield mainly through the effect on grain number; whilst grain weight was the component by which the area under the senescence curve affected positively yield (Fig. 4).
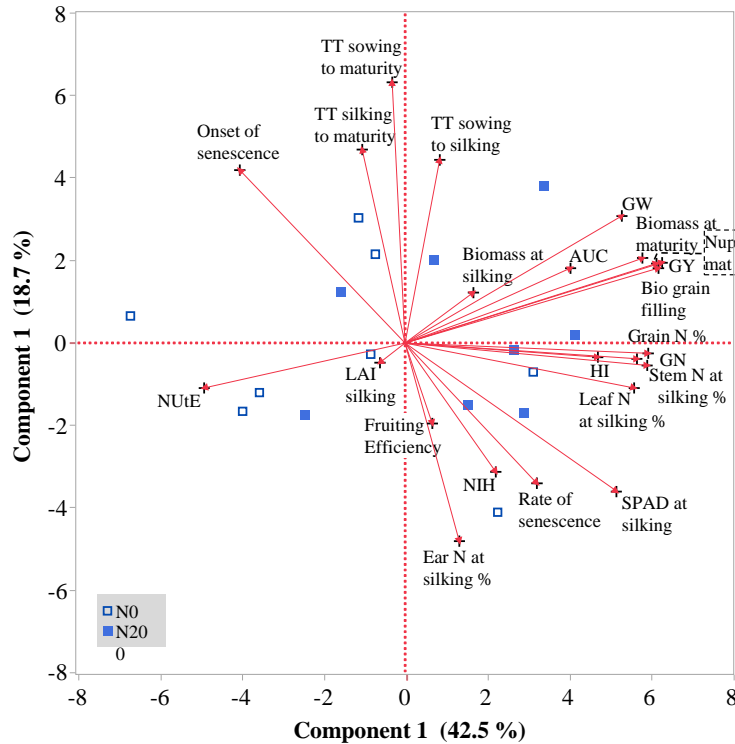
Figure 4. Biplot of the 1st and 2nd principal components for the two hybrids in the 8 environments. Variables are represent by letters, hybrids x environments by vectors, and N availability levels are identified by squares.

3.4 Discussion

Yield was not consistently related with cycle duration of the hybrids, which was similar between hybrids, *c.* 13 Mg ha^{-1} across all treatments and environments. Despite this general context, the relative response to N fertilisation tended to be higher in the long- than in the short-cycle hybrid, implying a higher plasticity may be expected in the long-cycle hybrid. This may be seen positively or otherwise depending on whether we are considering responsiveness to improved conditions or tolerance to stresses. Thus, analysing responsiveness in terms of the stability analysis it emerges that the PR31N28 tended to be potentially higher yielding whilst Lapopi tended to be more stable (Fig. 5).
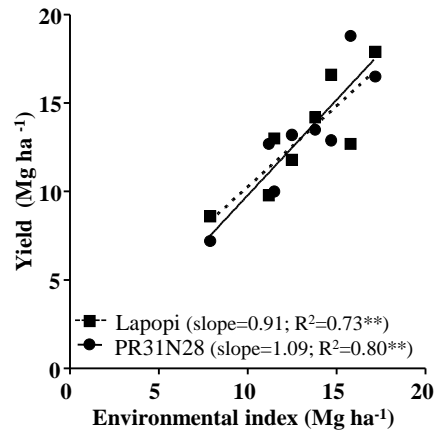
Figure 5. Yield stability analysis by regressing yield of each hybrid against the environmental index (average of yields in a particular environmental condition). Square symbols correspond to Lapopi and circles to PR31N28. Lines (dotted for Lapopi, plain for PR31N28) were fitted by regression.

Total biomass, LAI and total N uptake at silking was higher for the long-than the short cycle-hybrid at low and warm and similar at high and cold location. These differences found at low altitude disappeared at maturity, when all the values for these variables were similar in both cycle length hybrids. The end result was that both hybrids achieved similar yields in both locations, implying that for explaining the overall variation in yield growth after silking was more relevant than growth before silking. However, it cannot be discarded that part of the differences in growth after silking may respond to differences in sink strength (number of grains and potential size of the grains) that is set around silking (see detailed discussion and references in Chapters V and VI). The interplay between the determination of grain number and potential size of the grains, which might differ strongly between hybrids, may explain that in the present study both yield components explained yield similarly (though the range explored by grain number was a bit wider and therefore may be more responsible than grain size of the yield plasticity explored; see discussion on this issue in Sadras, 2007 and in Sadras and Slafer, 2012).

Expectedly, yield was related to the capture of resources than to the efficiency of use of these resources. This is generally the case when variation in resource availability is explored, like in the present study. It is normally the case that when crops are fertilised the growth is affected more than the partitioning and N uptake more favoured than N utilisation efficiency, which is normally reduced in response to improved availability.

Although, protein percentage is normally negatively related to yield. The reason for this negative relationship is that grain growth is largely sink limited while N accumulation in the grains is source limited producing a dilution effect (see Chapter VI for evidences and in depth discussion of the issue). However large changes in availability of resources may simultaneously modify sink strength (through improving grain number and potential size of the grains) as well as source of N (N content in vegetative organs which might be remobilised later), as illustrated in the bi-plot of the principal component analysis offered (in which GN increased simultaneously with N content in vegetative organs at silking) explaining the positive relationship overall conditions between yield and protein concentration of grains without conflicting with the idea that in concrete conditions N in the grains may be diluted by the amount of C being accumulated during grain filling. This is in fact consistent with that yield was in general related to N uptake and negatively related to N utilisation efficiency, being the later the driving force for the protein concentration in grains (the higher the efficiency in using a certain amount of N for producing yield the more diluted the N accumulated in the grains would be; e.g. Pedro et al., 2011).

Regarding to leaf area production, there were clear differences between locations. Values for this trait were lower in the warm than the cold environment (from *c.* 4.4 to slightly more than 5.1). Also, LAI values were different (P≤0.001) between hybrids, as also found other authors (Borrás et al., 2003; Maddonni and Otegui, 1996). However the differences between long- and short-cycle hybrids could be related to the fact that the long-cycle hybrid produced more leaves than the short-cycle, in agreement with what found since long time ago (Chase and Nanda, 1967). Senescence was clearly delayed by N fertilization. Leaf green area duration in the short-cycle was reduced in *c.*12 %, while in the long-cycle hybrid was *c.* 23 % under no N fertilised conditions. The response of leaf senescence rate (assessed as the loss of green colour) was more markedly in the low than in the high altitude location (Table 4; Fig. 3). This response probably could be explained based in the warmer temperature of low altitude during each growing stage than the high altitude. General senescence during grain filing is related to local growing conditions and the perceived light quantity by the leaf and nitrogen availability (Borrás et al., 2003). This effect was also observed by Tollenaar and Daynard (1978), in a comparison of 10 short-cycle hybrids during two consecutive years.

In conclusion, this detailed analysis of the short- and a long-cycle hybrids (which reasonably represent best adapted hybrids to the region; Chapter II)

(i) Corroborated, with a parallel but independent set of experiments, that long cycle hybrids may be a true option for the high altitude farmers (if they are prepared to assume a higher than usual risk of loses in exceptionally cold autumns), as well as the short-cycle hybrids may be a reasonably productive alternative for farmers in the plain of Lleida (and other similar environments within the Ebro valley) if they need to have their fields sown later (e.g. having tow crops per season) or released earlier for the same sowing time (e.g. harvesting with less moisture content in the grains, sowing a crop or a pasture earlier after harvest).

(ii) That N fertilisation normally improve yields but that in some cases the natural availability in soils may be more than enough (see also Berenguer et al., 2009; Cela et al., 2011), which is emphasised by the fact that when we were selecting fields for this study a requirement was that the field to be used were not broadcasted with slurry in the last seasons.

(iii) Overall the range of conditions yield was more strongly affected by capture, than by partitioning or efficiency of use of resources, and in this context grain yield was positively related to both of its components similarly (even though grain number was more determinant than grain weight for the plasticity of yield) as well as to grain protein concentration; but these relationships are actually driven by the huge environmental range (years x locations x N regimes) explored. In further chapters the physiology of determination of yield and the relationships with these other traits will be further explored.

(iv) In particular, the negative relationship between yield and NUE in the context of the wide range of conditions does not preclude the awareness that future hybrids shall be more NU Efficient and that ways to select for improved NUE must be developed for future agricultural systems in which N is expected to be less freely available while yields must keep increased. In the next chapter we tested a recently proposed surrogate for phenotyping for improved NUE taking advantage of the large range of GxE explored in Chapter 1.

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D

N:200 (100 y 100)

Chapter IV

Variation in the critical specific leaf nitrogen maximising yield among modern maize hybrids

4.1 Introduction

Further increasing yield has always been a major aim in breeding programs of grain crops. Large genetic gains in potential yield have been achieved in most major crops during the last century, though recent gains seem to proceed at a substantially slower pace than that required to match expected growing demands (Fischer et al., 2014; Ray et al., 2013). In maize, genetic gains in yield were mostly associated with increases in radiation use efficiency (RUE) (Fischer and Edmeades, 2010) and in tolerance to stresses, particularly population stress (Duvick, 2005; Luque et al., 2006; Tollenaar and Lee, 2006).

These increases in yield potential have been accompanied by large increases in the use of inputs, particularly nitrogen (N) fertilisation. A substantial proportion of the massive increase in yield production over the last 60-70 years has been attributed to improve crop management, in particular the amount of N fertiliser use (Duvick, 1992; Sinclair and Muchow, 1995). As modern agriculture must be increasingly concerned with environmental impact of crop management, the potential adverse impact of N fertilisation on environment must be minimized without affecting strongly the yield progress that is required. In this context, future genetic gains in yield would be expected to include improvements in N use efficiency (NUE).

Selecting for improved NUE would be unlikely in realistic breeding programs unless trustworthy surrogates assessed more easily and quicker than NUE could be identified. Substantial advances have been made in methodologies for phenotyping complex traits in maize breeding (Araus and Cairns, 2014), and many phenotyping tools have been identified (Cairns et al., 2012). Thus, if a trustworthy surrogate could be identified, it might be possible to phenotype for it in order to identify genotypes of putatively improved NUE. Around silking, when yield potential is being determined (Munaro et al., 2011; Paponov et al., 2005; Tollenaar et al., 1992), most plant N is allocated to leaves (Muchow, 1988; Tsai et al., 1991; van Oosterom et al., 2010). Then, for maximizing NUE leaf N at silking must be considered. Several papers have highlighted the critical role of specific leaf N (SLN, the mass of N per unit of leaf area) in maximizing crop growth during the critical period of silking, and thereby in maximizing yield (DeBruin et al., 2013; Massignam et al., 2011; Muchow and Sinclair, 1994; Sadras et al., 2000). The relationship between yield (or yield-determinant traits) and SLN is largely bi-linear: yield increases linearly with increasing SLN until a critical SLN

(SLNc) when yield is maximized and maintained over SLN values higher than SLNc. DeBruing et al., (2013) recently concluded that a SLN of 1.5 gN m^{-2} at silking is sufficient for maximizing yield (in the US Corn Belt). Therefore, it was proposed that it could be possible to phenotype populations to discard genotypes of SLN lower than this SLNc (DeBruin et al., 2013). For this assertion to be widely acceptable the range of variation in SLNc should be very small, at least among modern hybrids. Studies of the range of variation in SLNc are not abundant, as they require a number of genotypes being grown under a wide range of environmental conditions creating a large variation in SLN at silking for each of the genotypes. Thus, we aimed to determine the degree of genetic variation, if any, in a range of modern maize hybrids grown under contrasting growing conditions able to generate a wide range of both yield and SLN values for each hybrid.

4.2 Materials and methods

4.2.1 Experimental sites

Four field experiments were carried out at two contrasting locations in the province of Lleida (Catalonia; NE Spain) (i) the Plain of Lleida, Menarguens in 2009 (Lat. 41º 43' 55'' N; Long. 0º 44' 43'' E) and Algerri in 2010 (Lat. 41º 47' 41.2'' N; Long. 0º 38´ 52.6´´ E) representing a relatively warm environment in the Ebro Valley, and (ii) a valley in the Pyrenees, Montferrer in both 2009 and 2010 (Lat. 42º 20' 40.6'' N, Long. 1° 25' 47.4'' E) representing a relatively cold environment. All experiments were fully irrigated and pests, diseases and weeds were prevented or controlled.

4.2.2 Treatments and experimental design

All experiments were conducted under field conditions. Treatments included a factorial combination of 11 maize hybrids and two nitrogen levels. The hybrids represent all well adapted commercial hybrids with a range of maturity classes spanning from cycles characterized as FAO400 to FAO750 (Table 1). The two N treatments were an unfertilised control (N_0) and a heavily fertilised condition (N_{200}) in which urea was broadcasted at a rate of 200 kgN ha^{-1} when the plots had the sixth leaf visible.

Table 1. Maize hybrid used in this study and main characteristic described by the seed company.

Seed company	Hybrid	FAO cycle	Year of release	Characteristics (as described by the seed company)
Caussade semences	Lapopi	450	2003	Single cross hybrid, dent grain, diseases and fungus attack tolerance, early flowering.
Caussade semences	Paolis	650	2008	High yield potential, thick and homogeneous ear, lodging resistant, forrage and grain purpose, early flowering.
Caussade semences	Franki	400	2005	Simgle cross hybrid, semi dent, semi-precoss, excelent grain yield potential, early vigor, early-lodging tolerance, lodging resistance at harvest, stay-green, rot and fungus resistance, health conditions.
Euralis	Eurostar	400	2007	High stability and productivity, good adaptation to wide range of humid environmental conditions, early maturity.
Euralis	Earlystar	400	2006	Hight stability and grain potential yield, good quality for forage production, short cycle at phisiological maturity.
KWS	Kermess	700	2003	Mainly for grain production, stability and potential yield, vigorous and healthy plant, excelent stay-green, thick and restistant stems, high yield stability, ear rot resistance.
Monsanto	DKC6575	700	2003	Worm resistance (YieldGard gene), carbon resistance, heat stress tolerance, vigorous and healthy plant, stay-green, thick stem and strong root system, lodging resistance, good adaptability.
Limagrain	Beles Sur Bt	650	2006	Single cross,worn bore resistance (Bt gene), dent grain.
Limagrain	Guadiana	700	2006	Single cross, high yield, high leaf development, grain dent and lodgin resistance.
Limagrain	Helen Bt	750	2006	Sigle cross, high stability and yield potential, flowering semi-precoss, transgenic variety with (Bt gene), worn borer resistance.
Pioneer	PR31N28	700	2006	Provide with YieldGard gene and Bt gene, recomended for worm attack areas, good adaption in potential yield areas, good stability.

All experiments were arranged in a split-plot design with three replications, where hybrids were the main plots and N availabilities were the subplots. In the Plain of Lleida, the main plot consisted of 8 rows 20 m long with a distance between rows of 0.70 m. In the Pyrenees the main plot consisted of 8 rows 15 m long with a distance between rows of 0.75 m. In all experiments we sowed *c*. 96,000 seeds ha^{-1} and few weeks after seedling emergence we thinned the plots manually to warrant a uniform plant density of 85,000 ha^{-1}.

4.2.3 Measurements and determinations

Samples (2 m of a central row) were taken both at anthesis and at maturity. Plants were cut from ground level and weighed immediately in the field. A subsample of three plants per experimental unit was taken randomly from each of the samples and also weighed immediately in the field. Sub-samples were taken to the lab and processed separating stems (including leaf sheaths), leaves (actually leaf laminae), ears and grains (at maturity). The area of green leaves was determined in all subsamples using a Li-3100C area meter (Li-COR inc., Lincoln, NE, USA). Then all organs were oven-dried at 65 ºC for 72 hours and weighed. At physiological maturity, yield components were determined.

Nitrogen concentration was determined in the subsamples by Kjeldahl. After oven-dried, the plant tissues were grounded in analytical mills (one for vegetative tissues, another one for grains). Specific leaf area (SLA) was calculated by dividing the leaf area per plant by leaf mass, and SLN was calculated from the leaf dry weight.

Nitrogen utilisation efficiency (NUtE) was obtained as the quotient between yield and nitrogen uptake at physiological maturity; N harvest index was determined as the ratio between nitrogen content in the grains and in the whole above-ground biomass at maturity.

4.2.4. Analyses

Combined analysis of variance was performed for each variable in order to compare the relative importance of the main factors and their interactions for the main traits analyzed.

For the determination of SLNc, data of yield of each of the 11 hybrids were regressed against the corresponding values of SLN at silking, fitting the data to a bi-linear model (Y=

A+BX (X≤C) + BC (X>C): this model implies a linear increase in yield with increases in SLN until yield is maximized and does not keep increasing at higher values of SLN and therefore the slope of the second segment is forced to be zero. The breakpoint of this relationship (C) indicates the SLNc (e.g. the minimum SLN maximizing yield).

4.3 Results

As expected in studies conducted across different locations and with a number of genotypes grown under contrasting management (in this case N availability), virtually all traits were significantly affected by the main factors considered (year, location, hybrids and N) as well as by several of the possible interactions. However, in this study the magnitude of the direct effects (particularly those of the contrasting locations and N fertilisation levels) were in general consistently greater than the magnitude of the interactions (Table 2). In particular, there was a general consistency between the effects on yield and on SLN at silking, which is coherent with considering SLN a relevant physiological driver of yield.

Due to the different environmental conditions yields varied from less than 6 to more than 18 Mg ha^{-1} (Figure 1a, left panel), whilst SLN at silking varied from c. 0.7 to more than 2.5 gN m^{-2} (Figure 1a, right panel). Genotypic differences were naturally much smaller, as the genotypes tested were all currently commercial hybrids: the range in maximum yields (as a mean of the yield values corresponding to SLN at silking ≥ SLNc) ranged from c. 12 to c. 16 Mg ha^{-1} (Fig. 1b). Similarly the variation in maximum levels of SLN at silking ranged from c. 2 to slightly more than 2.5 gN m^{-2} (Fig. 1c).

Table 2. Mean square of each variables studied in two growing season (2009-2010).

Source of variation	DF	GY (Mg ha^{-1})	Biomass at maturity (Mg ha^{-1})	N uptake at silking (kg ha^{-1})	SLN (gN m^2)
Year	1	260.124**	538.794*	13177.954ns	10.116***
Location	1	405.265***	921.16**	169079.958***	3.323***
Year*location	1	79.781*	292.147ns	54544.719*	13.13***
Block (Year*Location)	8	10.705***	65.362***	6495.272***	1.01ns
Genotype	10	33.187***	221.137***	3547.461***	5.879***
Year*Genotype	10	8.702ns	60.75**	1967.757**	2.593***
Location*Genotype	10	9.280*	26.413ns	2508.507***	1.697*
Year*Location*Genotype	10	3.181ns	41.635*	2477.552***	1.004ns
Genotype*Block(Year*Location)	80	4.730**	19.203**	712.440ns	5.239ns
N	1	472.111***	1107.139***	176536.154***	11.649***
Year*N	1	6.563ns	10.837ns	51.959ns	0.076ns
Location*N	1	32.847***	82.389**	10559.425***	0.148ns
Year*Location*N	1	1.254ns	2.797ns	1487.485ns	0.000ns
Genotype*N	10	4.755ns	3.151ns	771.624ns	0.803ns
Year*Genotype*N	10	2.437ns	12.221ns	527.853ns	0.949ns
Location*Genotype*N	10	2.207ns	9.910ns	603.501ns	1.224ns
Year*Location*Genotype*N	10	3.160ns	9.270ns	1226.939ns	1.839*

Significant at the probability level of p *<0.05, **<0.01, ***<0.001; ns: not significant
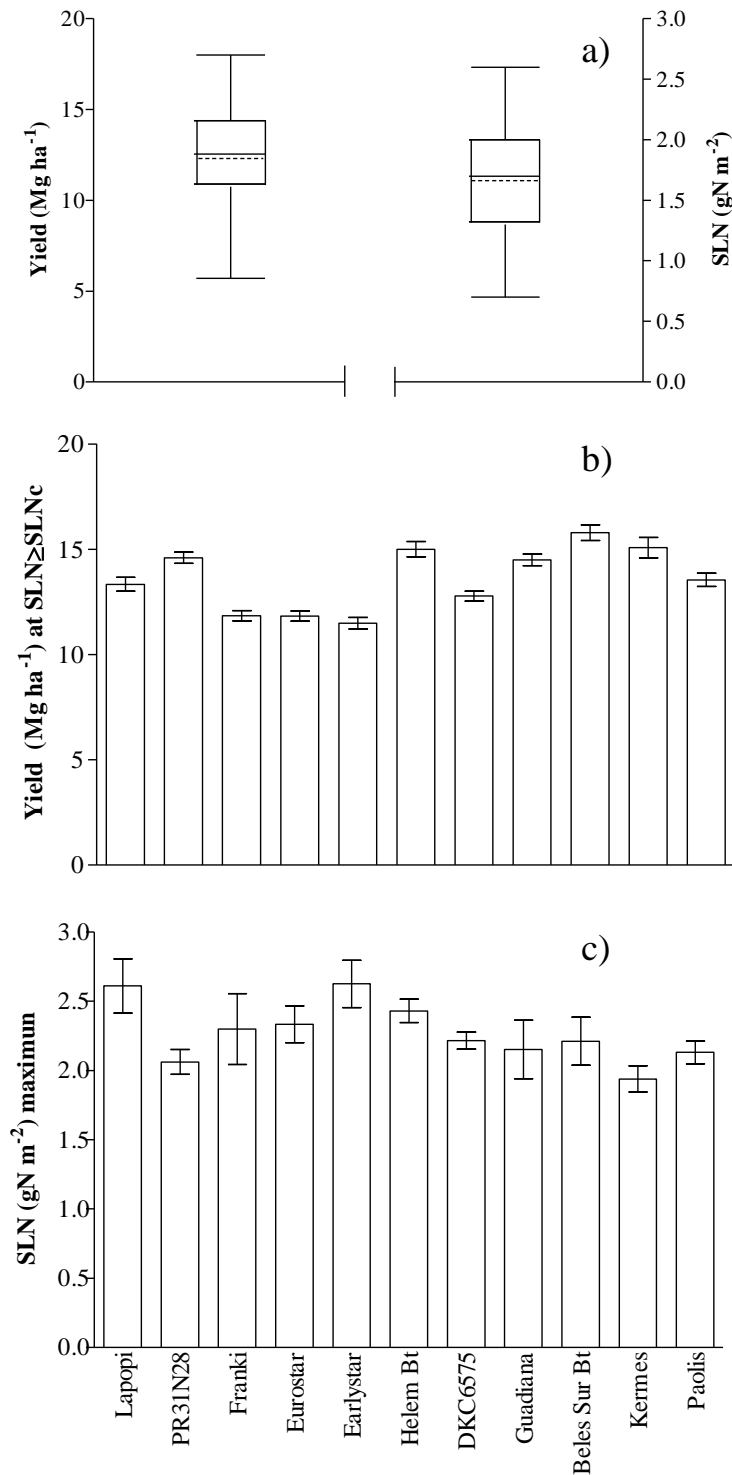
Figure 1. Boxplot of yield (a, left) and SLN at silking (b, right) observed in all environment by hybrid combinations; yield of each hybrid averaged across values observed above SLNc (b) and the maximum levels of SLN observed for each hybrid (c). Segments on top of each bar represent the standard error of the means

Pooling all the observations for yield and SLN together, the bi-linear relationship between yield and SLN at silking was significant, with an average maximum yield of c. 14 Mg ha^{-1} and an overall SLNc of 1.57 gN m^{-2} (Fig. 2).
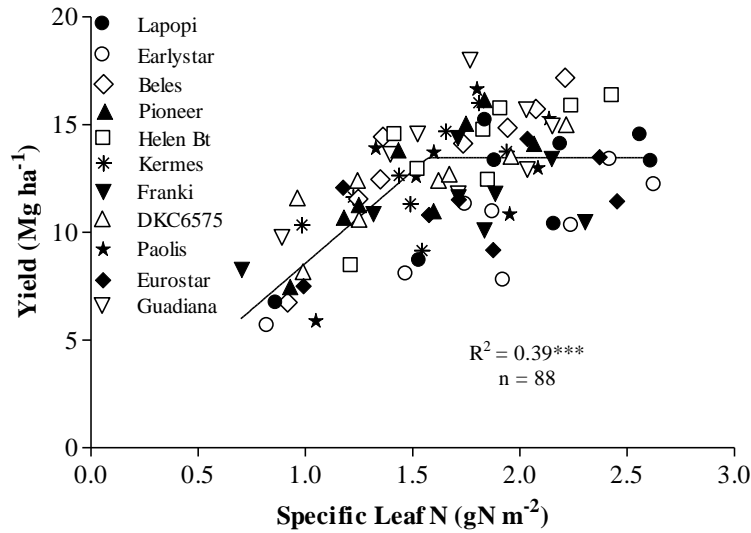
Figure 2. Relationship between yield and specific leaf nitrogen for the whole database (11 hybrids x 8 environments). Each point represents the mean values of 3 replicates.

The significant bi-linear relationship between yield and SLN at silking was also found for each of the 11 hybrids, as illustrated for Lapopi, the hybrid exhibiting an intermediate determination coefficient for this relationship (Fig. 3a), though the magnitude of the determination coefficient varied considerably from 0.21 ($P<0.01$) to 0.66 ($P<0.001$) (Fig. 3b). Most importantly, the hybrids varied quite significantly (both statistically and biologically) in SLNc, from 1.00 ± 0.14 to 1.92 ± 0.13 gN m^{-2} (Fig. 3b).

In an attempt to identify possible causes of the genotypic variation in SLNc, we related this threshold with several of the traits measured in the experiments for the different hybrids, considering the average of these traits for each hybrid for the conditions in which the SLNs observed were equal or higher than SLNc. Thus, we tried to identify if the values of a
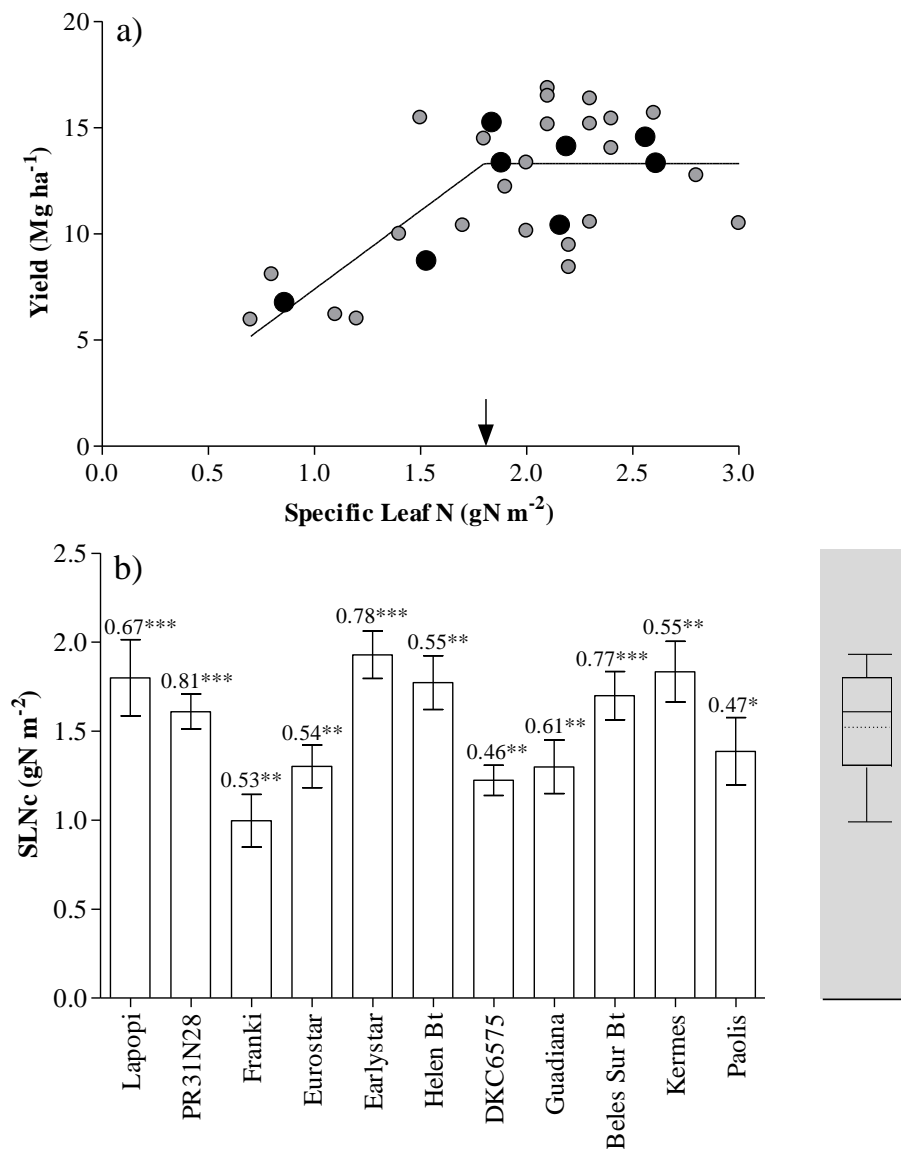
Figure 3. Bi-linear relationship between yield and SLN the hybrid "Lapopi" (which showed intermediate determination coefficient for this relationship) (a); and the critical values of SLN derived from these bi-linear relationship for each of the hybrids (SLNc: the minimum SLN maximizing yield) and an Boxplot representing the genotype variation in SLNc. Grey and black data points in panel a are the raw values of each observation and the average for the different environments respectively; the line represent the fitted bi-linear model, and the arrow on the abscissa stands for the SLNc. Figures and segments on the top of the bars in panel (b) stand for the correlation coefficient of the relationship from which each SLNc was derived (indicating with asterisks the significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$) and the standard error of the estimated SLNc respectively.

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particular trait under high-yielding conditions could explain the differences in SLNc. For instance there was a significant positive relationship of the SLNc and N uptake at maturity of the hybrids under high-yielding conditions (Fig. 4a), implying that in general hybrids with improved N uptake efficiency would also require higher concentrations of N in the leaves to maximize their productivity. However, the vast majority of the relationships were statistically not significant (Fig. 4b). Thus the differences in SLNc were largely independent of the yield potential of the hybrid.
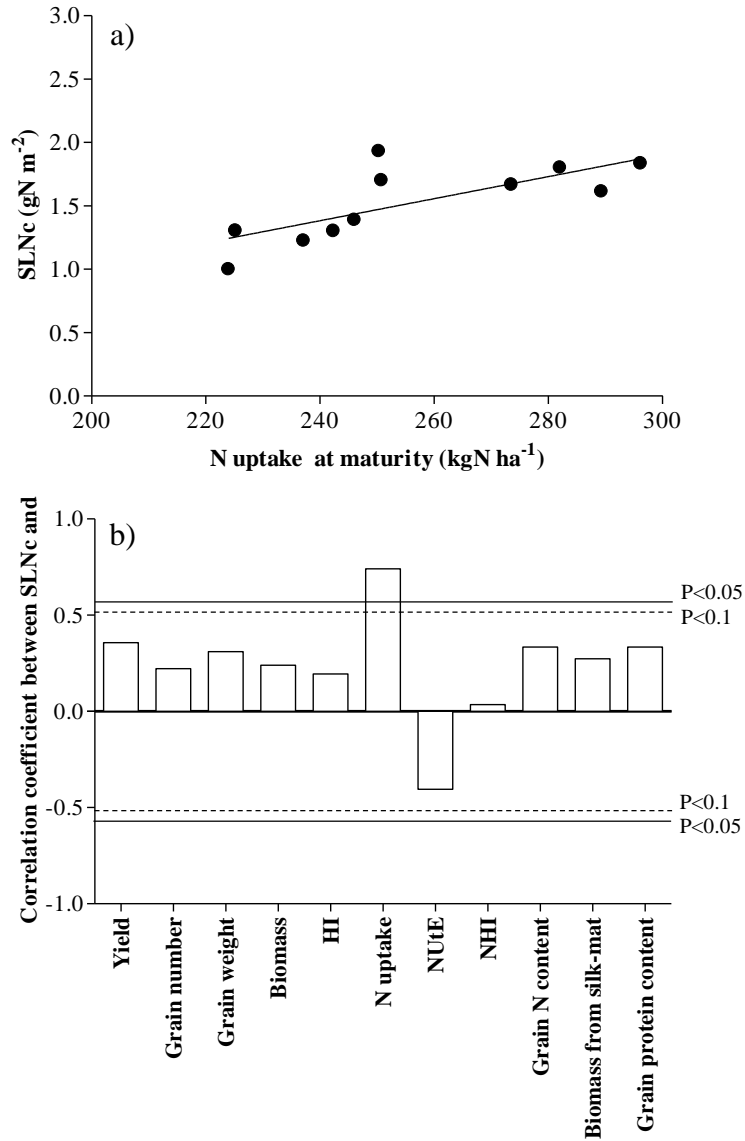
Figure 4. Relationship between SLNc and N uptake at maturity (average of conditions in which SLN≥SLNc) for the 11 hybrids (a); and correlation coefficient of the relationships between SLNc and yield as well as several determinants of yield, again averaging values for conditions in which SLN≥ SLNc (b). Dotted thick and thin lines represent the coefficients that are significant at P<0.05 and P<0.1 respectively.

4.4 Discussion

Identifying traits to phenotype populations for increased NUE would be relevant for future agricultural systems which are expected to yield more grain without increasing (or even with decreasing) use of inputs, such as N fertilisers. As much of the N absorbed by the crop

is in the leaves at silking (He et al., 2003; Rajcan and Tollenaar, 1999), the time when yield is largely determined (Lemcoff and Loomis, 1994); the relationship between yield and SLN at silking may be instrumental for identifying such phenotyping trait. Recently it was proposed that the critical SLN maximizing grain yield (a parameter derived from this relationship) would help phenotyping in commercial maize breeding, and from their analysis it was proposed that in low N environments a successful breeding program would focus on genotypes reaching at least a SLN of 1.5 gN m^{-2} at silking (DeBruin et al., 2013). This threshold for phenotyping was derived from relationships which pooled together 4 different hybrids grown under different regimes of N fertilisation. This threshold is actually very close to that we observed when pooled together the data of all the hybrids (in our study SLNc for all hybrids together was 1.57 gN m^{-2}).

However, for identifying a threshold for phenotyping with a complex trait like this it would be relevant that variation in this threshold were negligible; or at least that no genotypes exhibit a SLNc lower than the threshold for which the breeding program would be selecting for. Otherwise we may erroneously either (i) discard genotypes with SLN values lower than the threshold assuming they would not maximize yield in these conditions (for genotypes with a SLNc lower than the threshold proposed), or (ii) select genotypes with SLN values equal to the threshold assuming wrongly that they would maximize yield in these conditions (for genotypes with a SLNc higher than the threshold proposed). For that reason, determining whether there is noticeable genetic variation in SLNc is relevant to conclude on whether a threshold SLN can be used to phenotype to improve yield through increasing NUE.

We found substantial variation in SLNc (roughly from 1 to 2 gN m^{-2}) comparing 11 commercial hybrids. We did this comparison being conscious that the population analyzed would be very conservative but would represent fairly the type of material breeders work with when trying to improve yield, NUE or any other complex trait. It would be expected that a more variable population may express even a larger degree of variation in SLNc. Therefore, although it remains true that for improving NUE it would be very positive to select for genotypes with SLN = SLNc when grown under non-optimal N availability levels, there seems to be considerable intraspecific variation in SLNc and therefore it would

be hardly possible to use a particular threshold for SLN to phenotype plants which would be maximizing yield in those conditions.

Causes for the intraspecific variation in SLNc seem not simple. We related SLNc with yield in conditions in which SLN ≥ SLNc (the maximum achievable yield of the hybrid) and with a number of traits determining more or less directly achievable yield in the field and most of the relationships were not significant. Thus, the SLNc was rather independent of the hybrids differences in yielding ability. This could be related to variation among hybrids (when grown under contrasting N levels) in partitioning to the growing ear during a critical period for grain number determination in maize (D'Andrea et al., 2008). The lack of relationship would pose additional question marks on the appropriateness of phenotyping for a threshold in SLN presumably representing the SLNc, if the aim is improving NUE as a way to further increase yield potential. The only trait that was significantly and positively related to genotypic variation in SLNc was total plant N uptake. This would imply that hybrids with higher values of SLNc would require more N uptake and as there was no relationship between SLNc and achievable yield, hybrids of higher SLNc would tend to be less efficient in using absorbed N to produce yield. Although the magnitude of the correlation coefficient was not significant, the NUtE was the only trait negatively related to SLNc of the hybrids.

4.5 Acknowledgements

4.6 References

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Chapter V

Yield responsiveness to heat stress as affected by nitrogen

availability in maize

5.1 Introduction

Yield of cereals must increase dramatically in the next few decades. This is because the population will increase to some 10 billion people and the individual demands are growing simultaneously, and within cereals, maize demand would also increase noticeably due to the expected increase in its use in biofuel production towards 2050 (Fischer et al., 2014). These remarkable increases must be achieved in the context of a climate change which will imply that crops will be more frequently exposed not only to relatively higher temperatures but also to periods of heat stress (Battiste et al., 2009; Lobell et al., 2011; Cairns et al., 2013). Yield of cereals is impaired by higher temperatures during the growing season (Hatfield et al., 2011), but particularly when they occur during the most critical periods of yield determination.

Yield in maize is the consequence of the interaction between the number of grains and their average weight. Grain number is mainly determined during the critical period of 30 d bracketing silking (Andrade et al., 1999; Vega et al., 2001; Andrade et al., 2002; Westgate et al., 2004) when the number of grains per plant is determined in line with the rate of growth of the crop during that period (Vega et al., 2001) because it is during this period when the juvenile ear, where the female florets are developing, grow (Otegui and Bonhomme, 1998) and then the abortion process affects a proportion of the pollinated florets. Grain weight potential is largely determined during the same period (Gambín et al., 2006) and formally realised during the "lag phase" (Maddonni et al., 1998); but final grain weight is realised during the effective period of grain filling (Cirilo and Andrade, 1996; Borrás and Otegui, 2001).

High-temperature effects on yield may affect either of the two components, depending on the timing of occurrence of the heat (Rattalino et al., 2013). In principle, if the penalty imposed by the heat stress operates, at least partly, through reductions in crop growth, yield would be more affected when the heat occurs in the grain number determination period (around silking) as grain number determination is clearly source-limited (Gambín et al., 2006; Slafer and Savin, 2006) whilst grain weight seems more limited by the sink strengths (Gambín et al., 2008), at least if severe defoliations or very low levels of incoming radiation do not occur during the effective period of grain filling (Borrás et al., 2004). The

consequently higher responsiveness of grain number than grain weight to changes in resource availability (Slafer et al., 2014) explains why grain number is more plastic and grain weight more heritable (Sadras, 2007; Sadras and Slafer, 2012) and consequently yield is more related to grain number than to grain weight in most grain crops (Slafer et al., 2006), including maize (Otegui, 1995; Borrás et al., 2004). If the effects were not mediated by reducing crop growth, the magnitude of the penalty would be similar whether the stress occurs around silking or during the effective period of grain filling. It seems likely to hypothesise that high-temperature effects may be indirect, mediated by reducing crop growth (e.g. Cicchino et al., 2010b), though direct effects not mediated by reductions in growth are possible (Rattalino and Otegui, 2013).

A major inconvenience of studies aimed to uncover high-temperature effects on crop productivity is that, due to the difficulties in imposing the treatments under field conditions, they are most frequently conducted under controlled conditions. These studies are extremely useful for understanding detailed mechanisms of action of particular factors at relatively low levels of organisation. The problem is that results can hardly be extrapolated to field conditions (Passioura, 2006), where the practical consequences are expected. Scaling up from controlled conditions experiments to application in realistic field conditions may present several constraints (Passioura, 2010).

Recently a number of studies were conducted in the experimental field of the Univ of Buenos Aires by the group of Prof. Otegui enclosing for particular periods the maize canopy with transparent polyethylene film mounted wood structures build up *a priori* (Cicchino et al., 2010a; Rattalino and Otegui, 2011). A step forward in direction to increase the actual value of the conclusions to realistic system is to run such experiment in realistic farmer fields and in interaction with very common management practices, such as nitrogen (N) fertilisation, a step we pursued in this study.

Several agronomic and genetic strategies for increased tolerance to high temperatures will be necessary (Rosenzweig et al., 1994). The likelihood of mitigations through using plant growth regulators (Cicchino et al., 2013), or adequate management of magnesium (Mengutay et al., 2013) are being discussed. Around the world, food production increased linearly with the increment of nitrogen use in the agricultural systems (Tilman 1999), and N

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fertilisation is likely the most common management practice implemented in maize production worldwide. High yields in maize crop are closely associated with N fertilisation (Setiyono et al., 2010), mainly through affecting grain number (Carcova et al., 2000; Paponov et al., 2005) through modifying crop growth during the critical period around silking (Andrade et al., 2002; D'Andrea et al., 2008).

To the best of our knowledge, the interaction between heat stress and N availability has not been tested in maize. Both in wheat (Altenbach et al., 2003; Zahedi et al., 2004) and in barley (Passarella et al., 2008) it has been shown that the penalty on yield imposed by exposure to high temperatures were affected by the level of N availability: the higher the availability the more damaging the high-temperature effect (Altenbach et al., 2003; Zahedi et al., 2004; Passarella et al., 2008). If a similar sort of interaction were demonstrated, it may have relevant practical implications as in the future, when maize would be more often exposed to heat stresses, decisions on rates of N fertilisation should be taken not only considering the potential beneficial effects on crop growth but also a potential trade off on the magnitude of the penalty produced by heat stresses.

The main objective in this field study was to explore under field conditions whether the magnitude of yield penalty imposed by high temperature around flowering or during early grain filling is affected by the availability of nitrogen. In particular we aimed to (i) quantify the magnitude of yield losses by heat stress in these two phases, (ii) determine whether N fertilisation affects these magnitudes, and (iii) identify whether the effects are indirect (through affecting crop growth) or directly on the grain set and/or grain growth capacity.

5.2 Materials and Methods

5.2.1 General conditions

Field experiments were conducted on actual farmer's paddocks (rented for the experiments to be established using the normal sowing and management of the farmer, with the exception of the N fertilisation) close to Algerri ($41^{°}47^{′}41^{′′}$ N; $0^{°}38^{′}52^{′′}$ E), province of Lleida (Catalonia, north-eastern Spain) in 2009 (exp. 1), 2010 (exp. 2), 2011 (exp. 3), and 2012 (exp. 4), within the irrigated Mediterranean region of the Ebro River Valley. The

region has a semiarid continental climate, with low annual precipitations (374 mm annual, mostly in winter and early spring), low average air temperatures in winter (5.4 ºC), and high average air temperatures in summer (22.5 ºC) (Berenger et al., 2009). All experiments were sown within the normal sowing dates for the region and at a plant density within the range considered optimum (Table 1).

Table 1. Description of the general characteristics of the four field experiments.

| Year | Soil characteristics | | | Sowing date | Plant density (plants m^{-2}) | N treatments | Hybrid |
	OM* (%)	N-NO3** (kg ha^{-1})	P* (kg ha^{-1})				
2009 *Exp. 1*	1.9	175	70	12 May	8.026	N0	Lapopi
						$N200_{6H\dagger}$	
						N0	PR31N28
						$N200_{6H}$	
2010 *Exp. 2*	1.3	142	35.1	16 April	8.403	N0	Lapopi
						$N200_{6H}$	
						N0	PR31N28
						$N200_{6H}$	
2011 *Exp. 3*	1.5	225	72.1	31 March	8.403	N0	PR31N28
						$N100_{4H\dagger}$	
						$N100_{S-15\dagger}$	
						$N200_{4H}$	
						$N200_{S-15}$	
						$N200_{4H+S-15}$	
2012 *Exp. 4*	1.5	177	9.7	20 March	8.403	N0	PR33Y72
						$N100_{4H}$	
						$N100_{S-15}$	
						$N200_{4H}$	
						$N200_{S-15}$	
						$N200_{4H+S-15}$	

*Top 0.30 m of soil depth; ** top 1 m of soil depth
†4H, 6H and S-15 stand for V4, V6 and 15 d before silking, respectively. 4H+ S-15 means half was applied in V4 and the other half 15 d before silking.

All experiments were well irrigated (exp. 1 with sprinklers, exps. 2, 3 and 4 with drip irrigation). Water stress was prevented by sprinkler irrigation in the first year and drip

irrigation system near field capacity throughout the growing season in the other experiments. All the experiments were always kept free of weeds, pest and diseases by spraying recommended herbicides, insecticides and fungicides at the doses suggested by their manufacturers whenever necessary. Daily global radiation, minimum and maximum temperature, and precipitation (Fig. 1) were recorded at standard meteorological stations of the Agro-meteorological network of Catalonia, Spain, located close to the experimental fields.

5.2.2 Treatments and experimental design

Experiments 1 and 2. The treatments consisted in the factorial combination of two hybrids, two levels of N fertilisation and three temperature conditions. The genotypes were selected for representing single hybrids of different cycle duration but well adapted to the region. The short-cycle hybrid was Lapopi (classified as FAO 450) and the long-cycle hybrid was Pioneer 31N28 (PR31N28; classified as FAO 700). N fertilisation levels included only an unfertilised control (N0) and a fertilised treatment consisting on broadcasting N urea at V6 to a rate of 200 kg N ha^{-1} (N200) (Table 1). The temperature treatments consisted of a control (plots grown under natural temperature throughout the growing season) and two treatments in which the temperatures of the canopy were increased in the field: (i) from 15 days before silking to maturity (including the period of grain number determination as well as that of the effective period of grain filling), or (ii) from 15 d after silking to maturity (the whole effective period of grain filling) (Fig. 2a). Treatments were arranged in a split-split-plot design; the main plots were assigned to the two hybrids (Lapopi and PR31N28) to make the sowing practical, the sub-plots to the two nitrogen availabilities, and the sub-sub-plots to the temperature regimes. Main plots were 8 rows, 0.70 m apart, 40 m long; sub-plots were 20 m long; and sub-sub-plots were c. 1.5 m wide across 4 rows. There were three replicates arranged in blocks.
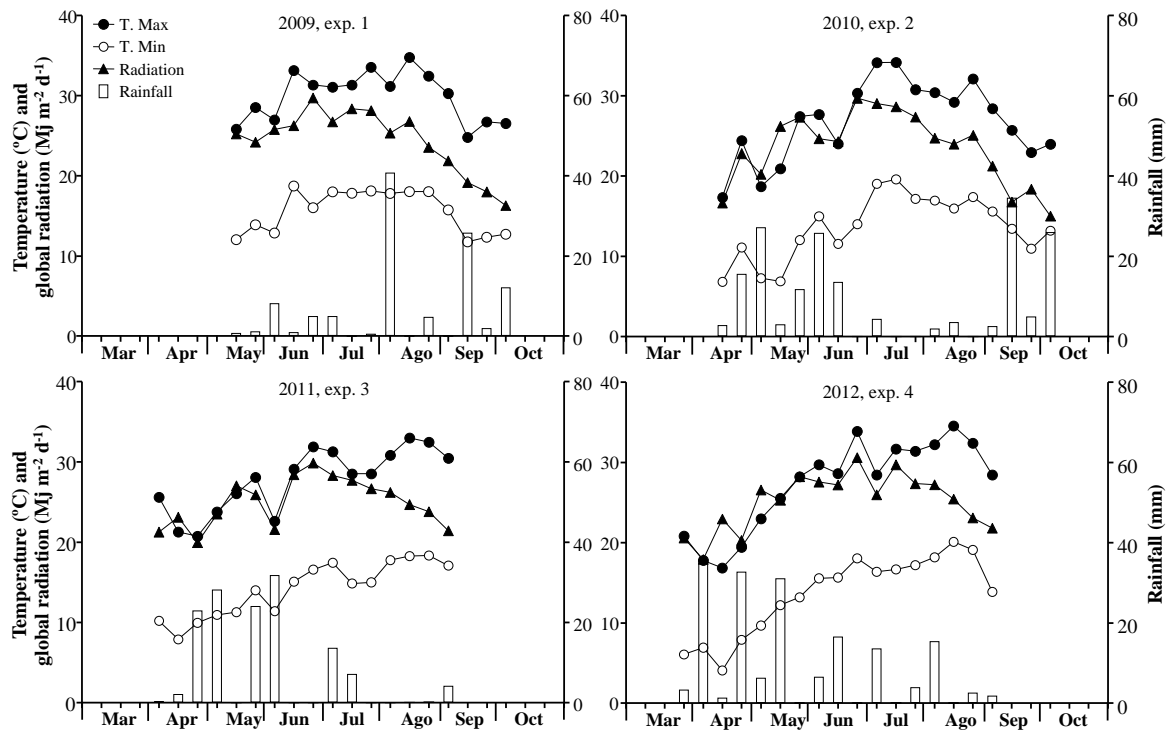
Figure 1. Mean global radiation (triangles), maximum temperature (closed circles) and minimum temperature (open circles), and accumulated rainfall (open bars) for each 10-d interval from sowing to maturity in field experiments carried out during 2009 (exp. 1), 2010 (exp. 2), 2011(exp. 3) and 2012 (exp. 4).

Experiments 3 and 4. The treatments consisted in the factorial combination of six levels of N fertilisation and three temperature conditions. N treatments combined three doses [0, 100, 200 Kg N ha^{-1}] and two timings [at V4 and at 15 days before silking] (Table 1). The temperature treatments consisted of a control (plots grown under natural temperature throughout the growing season), a high-temperature during the critical period (in this case it was from 7 d before silking to 9 d after silking at the beggining grain filling) and high-temperature during the first half of the effective grain filling period (from 14 to 32 d after silking) (Fig. 2a). In both experiments there was only one long-cycle hybrid (PR31N28 and PR33Y72 in exps. 3 and 4, respectively; Table 1). This was a limitation we had to assume in order to accommodate the several N treatments, and in this context we decided to use the long-cycle hybrid this type of hybrids are most commonly grown in the region and which had higher yields than the short-cycle hybrid in Exps. 1 and 2. PR33Y72 is the hybrid from Pioneer used by the farmer in 2012, following the advice of the Pioneer representative of

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the region. PR33Y72 is basically very similar to PR31N28 in all agronomic and physiologic traits we considered, including that they are both of the same maturity group (both classified as FAO 700). Treatments were arranged in a split-plot design; the main plots were assigned to the N fertilisation regimes, and the sub-plots to the temperature regimes. Main plots were 8 rows, 0.70 m apart, 20 m long; sub-plots were c. 1.5 m wide across 4 rows. There were three replicates arranged in blocks.

To impose the high-temperature treatment the designated area for the treatments was enclosed with transparent polyethylene film (100 μm thickness) mounted in wood structures of 3-3.5 m height (as illustrated in Fig. 2b), but leaving the bottom 30 cm of the four sides of each structure open, in order to have free gas exchange through that area. The magnitude of the high-temperature treatment was the consequence of the greenhouse effect of the polyethylene enclosure. Thus, differences produced by the enclosures were very clear for maximum temperatures and negligible for minimum temperature (Fig. 2 c, d, e). The air temperature was determined at the height of the tassels and the ears and recorded with a Em5b Anolog Data Logger (Decagon Devices USA). All the polyethylene films were installed at the beginning of each heating period and removed at the end.

As the way we imposed the high-temperature treatments increased markedly the maximum temperature of the tassels (Fig. 2c, d), which would have dramatically reduced pollen viability (Herrero et al., 1980; Schoper et al., 1987; Dupuis and Dumas, 1990), we hand-pollinated all the plants under heat stress during silking with fresh pollen collected daily from plants grown in unheated conditions. This has two consequences for the interpretation of our data: the cause of the effects of high-temperature treatments on grain number could not be attributed to lack of pollen or its viability; and the temperature increase that is most relevant for the conclusions is that of the ears.
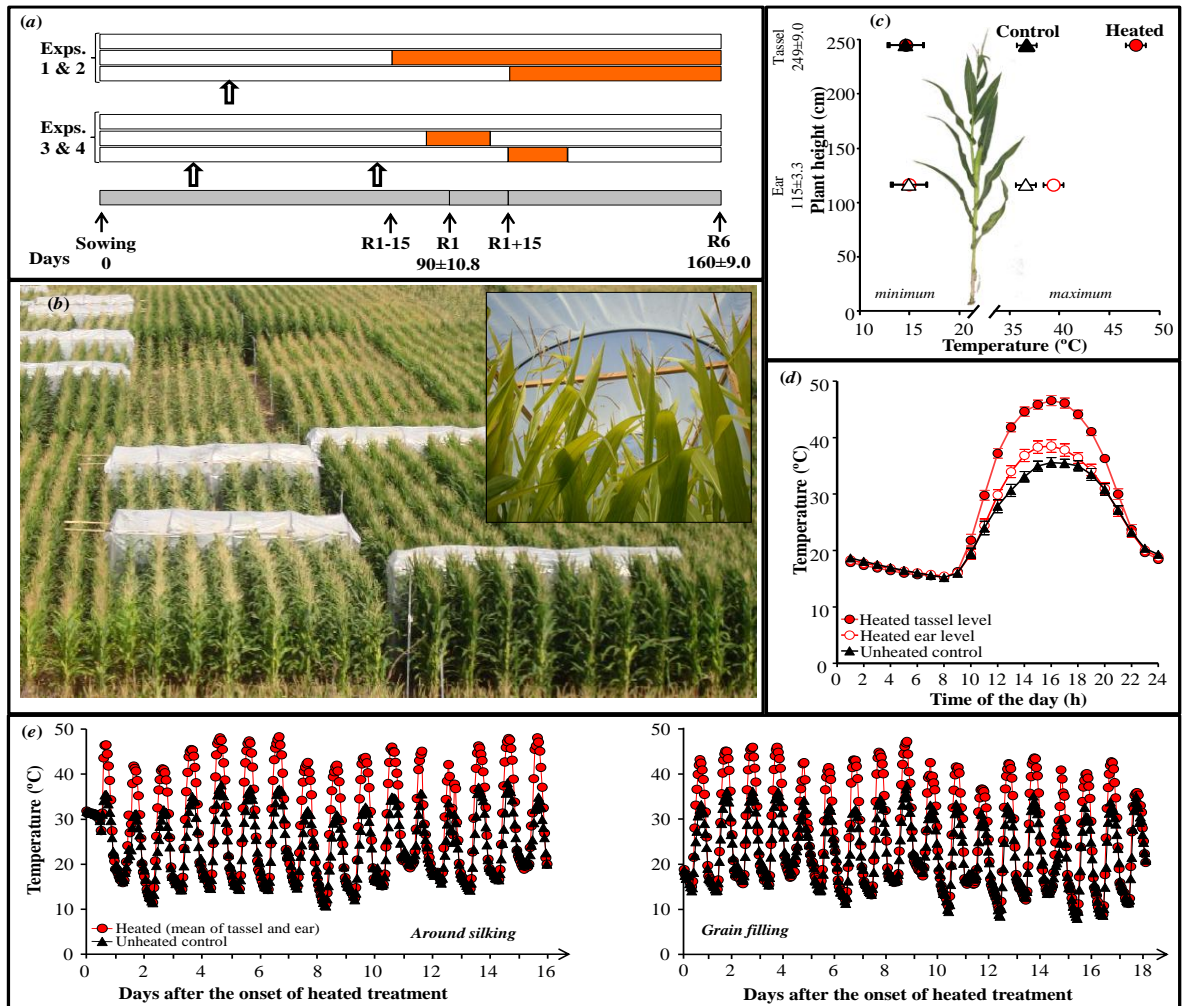
Figure 2. Description of the high-temperature treatments. *Panel a* shows the timing when treatments were imposed in each of the experiments (open bar, natural temperature; shaded bar high-temperature imposed). Open arrows indicate timing of fertilisation. The duration from sowing to silking (R1) and to maturity (R6) are indicated as an average (± the standard deviation; SD) of the different experiments and treatments. *Panel b* shows a partial view of exp. 1 with the high-temperature treatments imposed, with a detail on how the system looked like from inside the enclosures (top-right corner). *Panel c* shows the average minimum and maximum temperatures (left and right part of the panel) for the control (triangles) and for the heated treatments (circles) at the height of the tassels (closed symbols) and of the ears (open symbols) with the mean (±SD) height of these organs across treatments and experiments; the plotted temperatures are averaged across all days of treatments and experiments (bars stand for the SD). *Panel d* shows the average hourly temperature during the periods of treatment imposition for the control (triangles) and for the high-temperature treatments at the height of the tassels (closed circles); and the ears (open circles); temperatures averaged for each hour of the day across experiments and treatments, bars stand for the SD. *Panel e* shows the example of the hourly dynamics of temperature for the unheated control (triangles) and high-temperature treatments (averaged for temperatures at the height of ears and tassels, circles) imposed at around silking (left) or during the effective period of grain filling (right) in exps 3 and 4.

5.2.3 Sampling and measurements

There were two distinct plant sampling procedures: one for the treatments under unheated conditions, in which the size of the experimental units was very large, and another one for the heated sub-sub-plots (exps. 1 and 2) or sub-plots (exps. 3 and 4), in which the availability of plants was restricted, when proper borders were taken into account. For the treatments under unheated conditions, in early stages of development we inspected the crop stands and the areas for sampling (two rows, 1 m long; i.e. 1.4 m^2 including *c*. 11-12 plants per experimental unit per sample) at silking and at maturity were selected and labelled. The selection was based on having the exact density of plants uniformly distributed both in the area to be sampled and in the borders of those areas. In these conditions virtually all plants were identical within each hybrid and N regime. For the high-temperature sub-sub-plots (exps. 1 and 2) or sub-plots (exps. 3 and 4), including those of the unheated control, we identified at the same time several individual plants that were, within each hybrid and N regime, comparable to those of the two sampling areas of 1.4 x 1 m^2 labelled for the samplings at silking and maturity (*i.e.* exactly the same developmental stage, leaf number, and plant size; and being at the same plant density and uniformity). Just before the imposition of the first high-temperature treatment (*c*. 15-20 d before silking) we reselected from these labelled plants the three which, at that advanced pre-tasseling, stage were indistinguishable in development and growth aspects from those of the large sampling areas within each hybrid x N regime.

In each experimental unit we determined the timing of anthesis and silking when the labelled plants in that unit were shedding pollen from the tassels and emerging stigmas (silks) from the husks of the ear, respectively. The timing of maturity was determined by periodic inspection at the end of the grain filling period when the black layer was formed. When the duration of developmental phases was expressed in thermal time, we used a base temperature of 8ºC (Cirilo and Andrade, 1996).

From the samplings at silking and maturity we separated the leaf laminae, the stems (including the leaf sheaths and tassels) and the ear. These organs were oven-dried for 72 h at 65ºC and weighed. At maturity grains were threshed and counted and the yield components determined. N content was determined using the Kjeldahl method to the milled

samples of leaf, stems and grains. The three individual plants labelled in each experimental unit were non-destructively measured once or twice a week from 15 d before to 15 d after silking and biomass accumulation during the critical period for grain number determination was estimated based on allometric relationships (Vega et al., 2001). These relationships were derived from conventional plant samplings across different treatments and timings in all the experiments in which we measured morphometric variables (plant height, stem diameter at the base of the plant, length and diameter of the base of the ear; allowing the assessment of stem plus ear volumes, as in D'Andrea et al., 2008) as well as above-ground dry weight through oven-drying the samples for 72 h at 65ºC. We then used the calibrated allometric model (r = 0.84 P<0.001) to assess biomass in the non-destructive determinations made in each of the three plants of each experimental unit of each experiment. This approach to estimate plant biomass non-destructively has been successfully used in several other studies including potential and stressful conditions (e.g. Vega et al., 2001; Borrás and Otegui, 2001; Echarte and Tollenaar, 2006; Pagano and Maddonni, 2007).

Chlorophyll content of the ear leaf was estimated with SPAD (chlorophyll meter SPAD 502, Minolta Co. Ltd., Japan). SPAD readings were taken from silking to maturity once a week on four points (central portion) of the ear leaf in each treatment of all the experiments; and the dynamics of SPAD values through grain filling determined.

5.3 Results

5.3.1 Time to silking

The duration of the pre-silking period varied between the experiments. It was, averaged across all treatments in each experiment, 68.4±1.9 d, 79.1±1.9 d, 85.6±0.7 d and 98.5±1.2 d in exps. 1, 2, 3 and 4, respectively (Fig. 3). The difference simply reveals the differences in time of sowing between treatments (each experiment from 1 to 4 was sown earlier than the previous one; Table 1). Also the relatively small standard deviations imply that only small effects were produced by the treatments in time to silking; and this deviation was consistently higher in exps. 1 and 2 than in exps. 3 and 4, due to the use of hybrids of

different maturity groups (FAO 450 and 700) in the former two experiments while using a single hybrid in each of the last two experiments.

Exp. 1 exhibited much lower differences in time to silking between the hybrids than that observed in exp. 2 (cf. Fig. 3 upper left panel and Fig. 3 upper right panel). In exp. 1, only the FAO 700 hybrid was longer than the FAO 450 hybrid under high N availability, and even in this case the difference in cycle to flowering was marginal (c. 4 d; Fig. 3 upper left panel); while in exp. 2, PR31N28 was not only consistently longer than Lapopi but also the magnitude of the difference was more noticeable (c. 10 d under both N availabilities; Fig. 3 upper right panel). Disregarding the hybrid differences, all other treatments seemed to have negligibly affected time to silking.
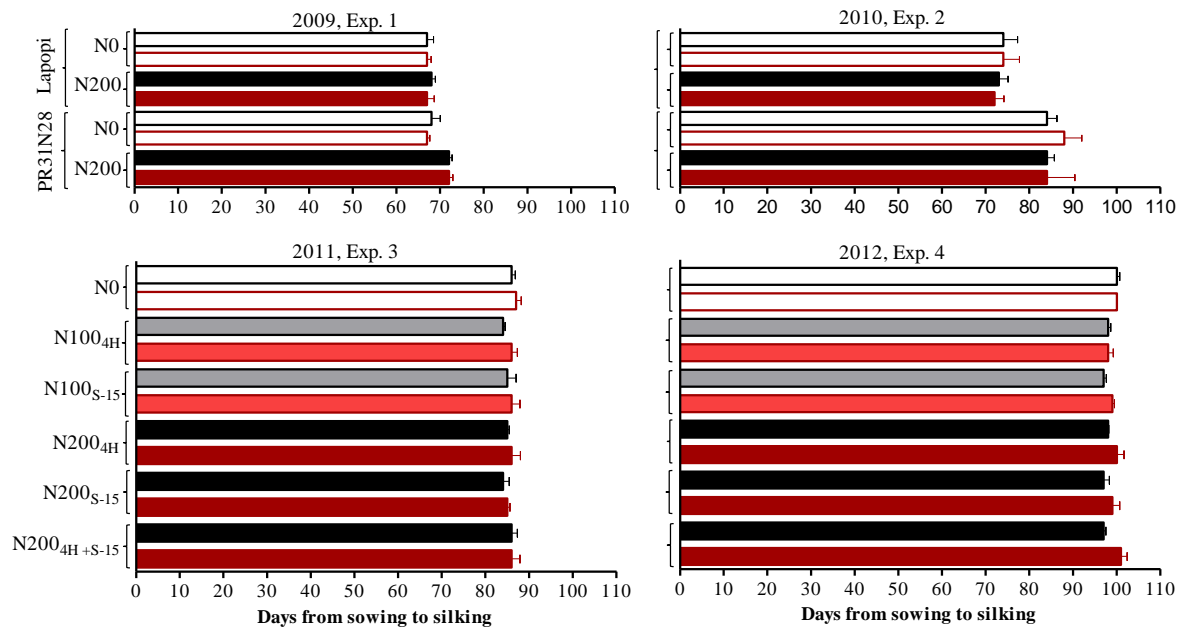
Figure 3. Duration of the pre-silking period for each combination of hybrid (in exps. 1 and 2) and N level (N0 open bars; N200 closed bars; N100 intermediate intensity) for the unheated control (black) and the treatments heated from pre-silking (the high-temperature treatment started 15 d before silking in exps. 1 and 2 and 7 d before silking in exps. 3 and 4; red). For details on fertilisation nomenclature please see Table 1. Bars stand for the standard error of the means. Each bar is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

There were not consistent effects of N availability and any of the effects were marginal (Fig. 3). For instance, higher N availability tended to delay silking in PR31N28 (Fig. 3 upper left panel) but tended to accelerate silking in exp.2 (Fig. 3 upper right panel) and there was not a consistent pattern of the different N fertilisation regimes compared to the unfertilised control in exps. 3 and 4 (Fig. 3 lower panels)

We expected some consistent acceleration of development, advancing silking, by imposing the high-temperature treatment (not very large due to the fact that the duration of the treatment was short; 6-15 d before silking), but the treatment did not accelerate development in any of the experiments (Fig. 3). In fact, it occasionally tended to delay time of silking, particularly in exps 3 and 4 (averaging across all hybrid x N treatments of the four experiments silking was delayed by 0.7±1.3 d, and averaging across all N treatments of exps. 3 and 4 the delay was 1.1±1.0 d), though the overall trend was not significant (Fig. 3).

5.3.2 Yield

Due to limited area available within the enclosures used for imposing the high-temperature treatments we measured yield and all other traits in three individual plants per experimental unit. Even though these plants were meticulously selected to be identical in development and growth aspects and in the same competitive environment to those of the large sampling areas in the unheated conditions, we prefer to report our results on a "per plant" basis (though in some cases, we indicated the corresponding values on a "per ha" basis for reference).

Yield of Lapopi, the short cycle hybrid, was consistently lower than that of the long cycle hybrid, PR31N28 (Fig. 4 upper panels). Also responsiveness to N in the unheated conditions was smaller in Lapopi than in PR31N28 (c. 5 and 35% averaging across exps. 1 and 2, respectively). The long cycle hybrids did also respond clearly to N in exps. 3 and 4 (Fig. 4 lower panels). In both exps. 3 and 4, the response to N was larger than that observed for the long-cycle hybrid in exps. 1 and 2 (cf. Figs lower panels and 4 upper panels). In general in these experiments the response was proportional to the rate applied (yield was

largest in N200 and intermediate in N100) if the plots were fertilised early (either fully; N200$_{4H}$ or at least half of the full dose was applied early N200$_{4H+S-15}$). If no N was applied until close to the reproductive stages (N100$_{s-15}$ or N200$_{s-a5}$) the response was clearly lower than with the early application in exp. 3 (Fig. 4lower left panel) but not in exp. 4 (Fig. 4 lower right panel).
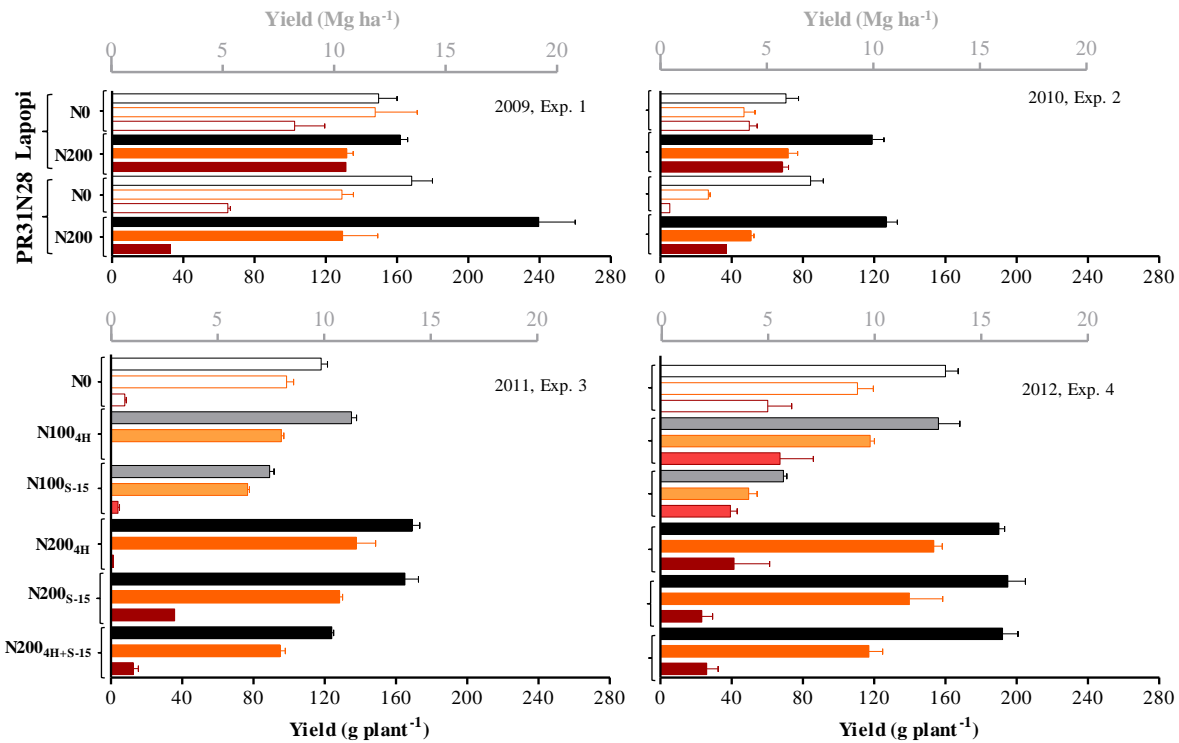
Figure 4. Yield of each combination of hybrid (in exps. 1 and 2) and N level (N0 open bars; N200 closed bars; N100 intermediate intensity) for the unheated control (black) and the high-temperature treatments starting either two weeks after silking (orange) or before silking (the high-temperature treatment started 15 d before silking in exps. 1 and 2 and 7 d before silking in exps. 3 and 4; red). For details on fertilisation nomenclature please see Table 1. Bars stand for the standard error of the means. An axis of yield per unit land area was also included in grey. Each bar is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

Disregarding that the high-temperature treatment only increased the maximum temperature (the minimum was unaffected; Fig. 1) in a relatively small magnitude (so that the mean daily temperature was increased at the height of the ears by *c*. 1ºC each day of treatment), the yield penalty imposed by the heat stress was very noticeable in general, and dramatic

when the treatment included the critical period for grain number determination, around silking (Fig. 4). The damage was also much stronger in the long- than in the short-cycle hybrid, particularly when the treatment was imposed before silking (Fig. 4 upper panels).

Yield was much more severely penalised by the heat stress when the high-temperature treatment started before silking than when it was restricted to the effective period of grain filling. After observing the results of the first two experimental years we supposed this was so due to the fact that the treatment starting earlier also lasted much longer in exps. 1 and 2 (Fig. 1, upper right panel), and it is normally the case that the longer the duration of a particular stress the higher the loss in yield produced. We then changed the treatments in the last two experiments for them to have similar duration but only differ in the timing, and the high-temperature treatments starting before silking produced very similar yield loss to that produced by the treatments starting before silking in the first two experiments (Fig. 4), albeit that in exps. 3 and 4 the duration of the heat stress was rather limited (Fig. 1). In fact the yield penalty imposed by high-temperature treatments stressing the crop for only 3 weeks around flowering in exps. 3 and 4 was much higher than the penalty imposed by the high-temperature treatments for more than 6 weeks when the grains were filling in exps. 1 and 2 (Fig. 4).

The main focus of this study was to test whether the magnitude of yield penalty imposed by high temperature around flowering or during early grain filling is affected by the availability of nitrogen: in other words if there was a pattern for an increase in damage by the same heat stress when crops were grown under higher N availabilities. Due to differences in soil N availability at sowing (Table 1) as well as in rates of mineralisation during the crop growing season (not determined) it is impossible to establish such relationship directly. As all experiments were fully irrigated and protected against biotic stresses, it can be trusted that yield is in fact mainly a function of N availability, and
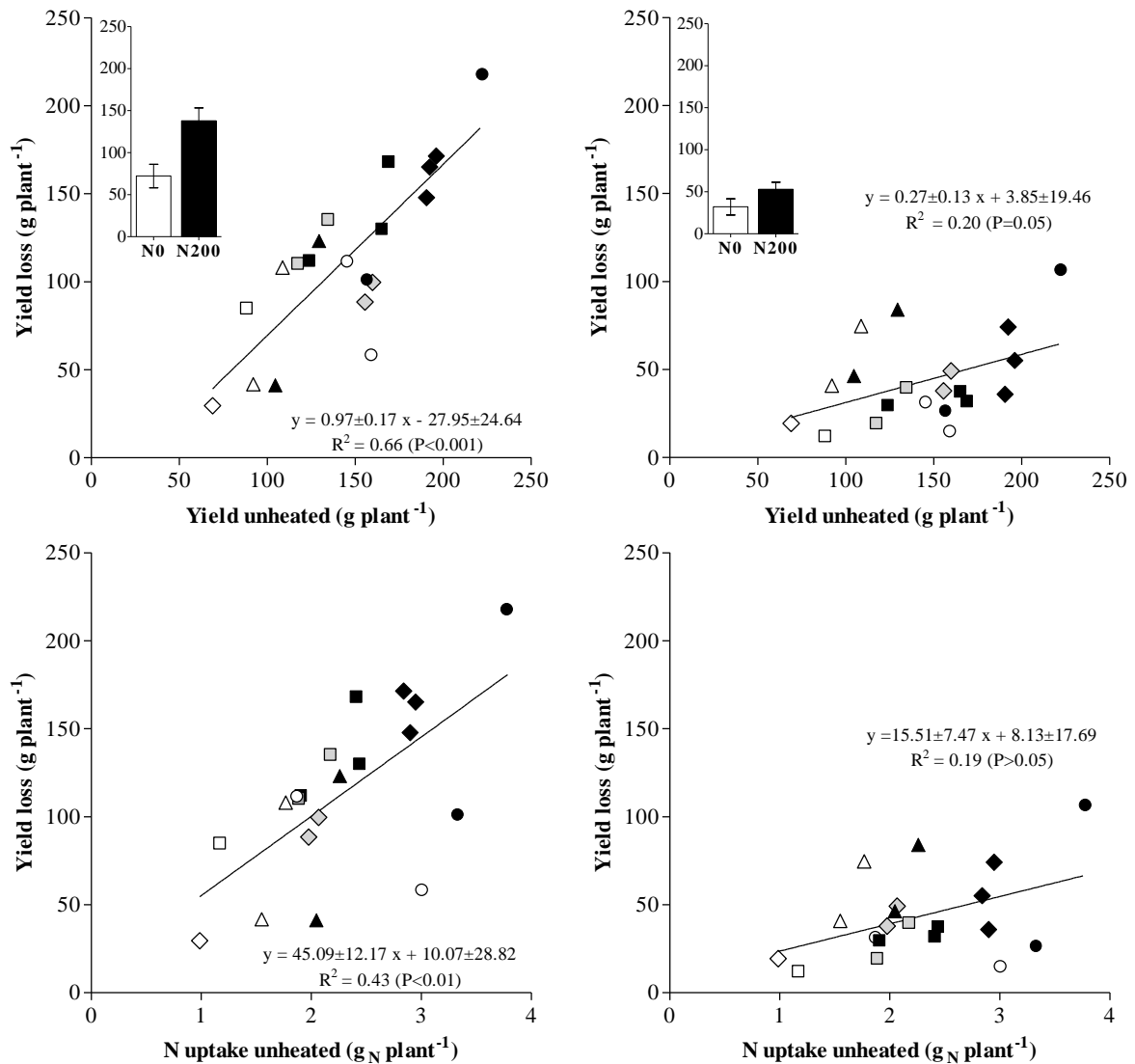
Figure 5. Relationship between yield loss due to the imposition of high-temperature treatments and yield under unheated conditions under different N availabilities (N0, open symbols; N100, grey symbols, N200, black symbols) across all experiments (exp. 1, circles; exp. 2, triangles; exp. 3, squares; exp. 4, rhombuses). High-temperature treatments started either before silking (15 or 6 d before in exps. 1 and 2 or 3 and 4, respectively) affecting the critical period for grain number determination (left panel) or at the onset of the effective grain filling period, 15 d after silking (right panel). Lines were fitted by linear regression. Inset is a summarised description of yield losses at the extreme N fertilisation doses, averaged for all the cases with each dose; bars stand for the standard error of the means. Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment). The two bottom panels are the relationships equivalent to the top panels but using N uptake in the unheated conditions as the independent variable instead of yield.

therefore we related yield loss due to heat stress for each N fertilisation regime (identifying the different N doses) with yield under unheated conditions across all experiments (Fig. 5).

Indeed there was a positive relationship between the magnitude of the yield loss due to the imposition of the heat stress and yield under unheated conditions (Fig. 5). The relationship was much sharper (higher slopes and coefficients of determination) when the treatments were imposed earlier, affecting the critical period for grain number determination directly, than when the treatments were most directly affecting the crop during the effective period of grain filling (cf. left and right top panels of Fig. 5). Even within particular experiments it was generally true that the higher the N availability the larger the losses imposed by heat stress. Consequently, averaging across experiments, the plots fertilised with N200 showed the largest, and those unfertilised the lowest, yield loss when affected by heat stress (Fig. 5 top panels, insets), and the N100 treatments had losses intermediate to those of N0 and N200. The effect was much clearer for the treatments affecting the critical period of grain number determination than for the grain filling (Fig. 5, top panels insets).

The high-temperature treatments imposed before silking did produce a collapse of the yielding capacity of the crop: the slope of the relationship was not different from 1 and the intercept not different from the origin, implying that in most cases the heat stress caused a situation close to sterility (Fig. 5, left top panel).

Although the linear relationship was highly significant, the distribution of the data-points of the treatments corresponding to N0 and N200 were not randomly distributed along the regression fitted: the residuals of the data-points for the highest yields in the unheated control (N200) were in average positive and those for the lowest yields in the unheated control (N0) were in average negative. Consequently yield loss was in general much larger under N200 than under N0 conditions, as depicted in the inset bar graph (inset Fig. 5, left top panel).

As using N uptake instead of yield as the independent variable did not improve (it actually worsened) the relationships (Fig. 5, bottom panels), it seems likely that the effects of N would not be direct but through affecting plant growth.

In fact, the high-temperature treatments imposed before silking produced a penalty in yield well beyond the impact it had on growth. Total biomass was reduced by approximately a half of the unheated controls whilst yield was reduced to c. one fourth of the controls if not directly collapsed (Fig. 6, left panel).
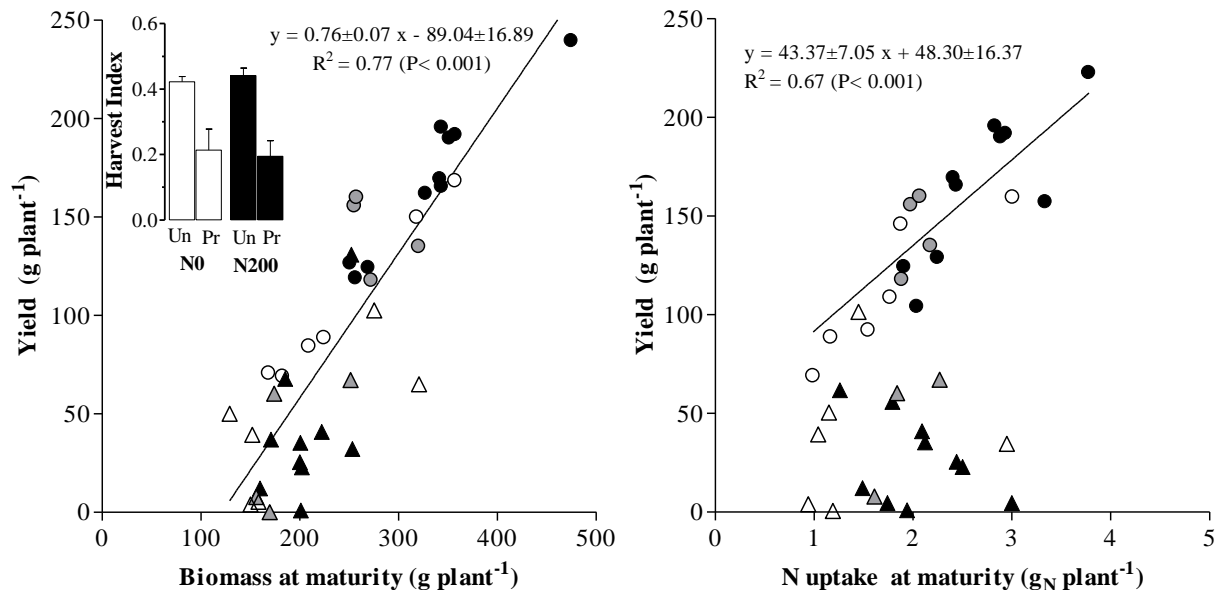
Figure 6. Relationships between yield and biomass (left panel) or N uptake (right panel) at maturity for high-temperature treatments starting before silking (triangles) and an unheated control (circles) in factorial combination with different N fertilisation regimes (N0, open symbols; N100, grey symbols, N200, black symbols). Lines were fitted by linear regression considering all data-points (left panel) or only the data corresponding to the unheated controls (circles, right panel). Inset on the left panel is the harvest index for the extreme N fertilisation doses, averaged for all the cases with each dose, and the temperature treatments (the unheated control, Un; the heat stress starting before silking, Pr); bars stand for the standard error of the means. Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

Therefore, exposing the plants to high-temperatures during the critical period dramatically reduced harvest index, from values of around 50% in unheated conditions to less than half under high-temperature, being the effects again more noticeable under N200 than under N0 fertilisation treatments (inset Fig. 6,left panel).

As the dynamics of N uptake is advanced respect that of dry matter accumulation, the imposition of the treatments affected N uptake much less than biomass and consequently

analysing the effects of the high-temperature treatments imposed before silking in terms of N utilisation efficiency the fall is even greater than that produced in harvest index (Fig. 6, right panel). Again the loss of yield (or even its collapse) caused by pre-silking heat stress imposition was well beyond the effects of the treatment on impairing N uptake.

5.3.3 Yield components: grain number

Yield was strongly related to the number of grains. Not only was the overall relationship significant but that for each of the thermal conditions was significant as well (Fig. 7, left panel). The number of grains per plant was also the main component affected by the high-temperature treatment, not only when the stress started before silking but also when it started 15 d after silking (Fig. 7). The latter treatment, imposed during grain filling (either the whole effective grain filling period or the first half of it, exps. 1-2 and 3-4, respectively) did also affect the average weight of the grains. Thus the main component affected by both treatments was the number of grains without compensations in the weight of the grains (Fig. 7, right panel).

As with yield, the magnitude of the reduction in grain number due to high temperature when the stress started before silking was higher in the fertilised than in the unfertilised condition, particularly when the stress started in pre-silking (Fig. 7, inset of left panel). The effects of either the high-temperature or the N treatments on the number of grains did not bring about a clear compensation in the average weight of the grains (Fig. 7, right panel). In general there was no major effects of high-temperature treatments starting before silking on the average grain weight, and when the heat stress was focused on the effective period of grain filling, there was a clear reduction, though the magnitude was not large (see data-points corresponding to post-silking heat stress consistently below the overall relationship on left panel of Fig. 7, and the inset of right panel).
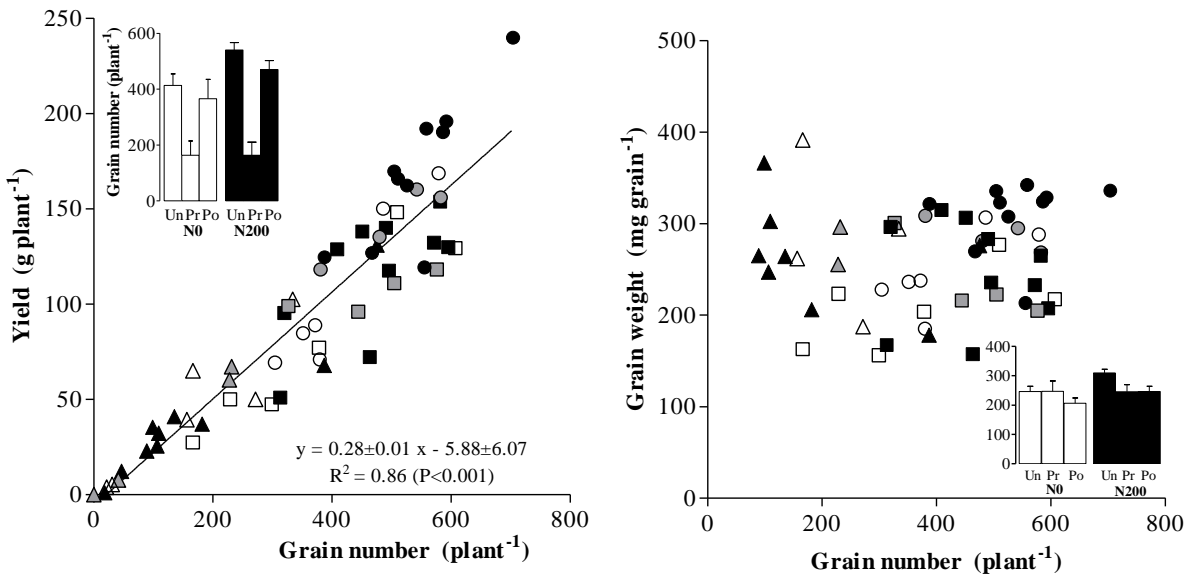
Figure 7. Relationships between yield (left panel) or average grain weight (right panel) and the number of grains for high-temperature treatments starting either before (triangles) or after silking (squares) and an unheated control (circles) in factorial combination with different N fertilisation regimes (N0, open symbols; N100, grey symbols, N200, black symbols). Line in left panel was fitted by linear regression. Inset are the number of grains (left panel) or the average weight of the grains (right panel) under the extreme N fertilisation doses, averaged for all the cases with each dose, and the three temperature treatments (the unheated control, Un; the heat stress starting before silking, Pr; and the heat stress starting 15 d after silking, Po); bars stand for the standard error of the means. Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

The number of grains was related to the plant growth rate during the critical period. A single overall relationship for all the conditions affecting growth during this period of 30 d bracketing silking (regarding N, all treatments; regarding high-temperature, only the unheated controls and the treatments starting before silking) was significant (Fig. 8, left panel).
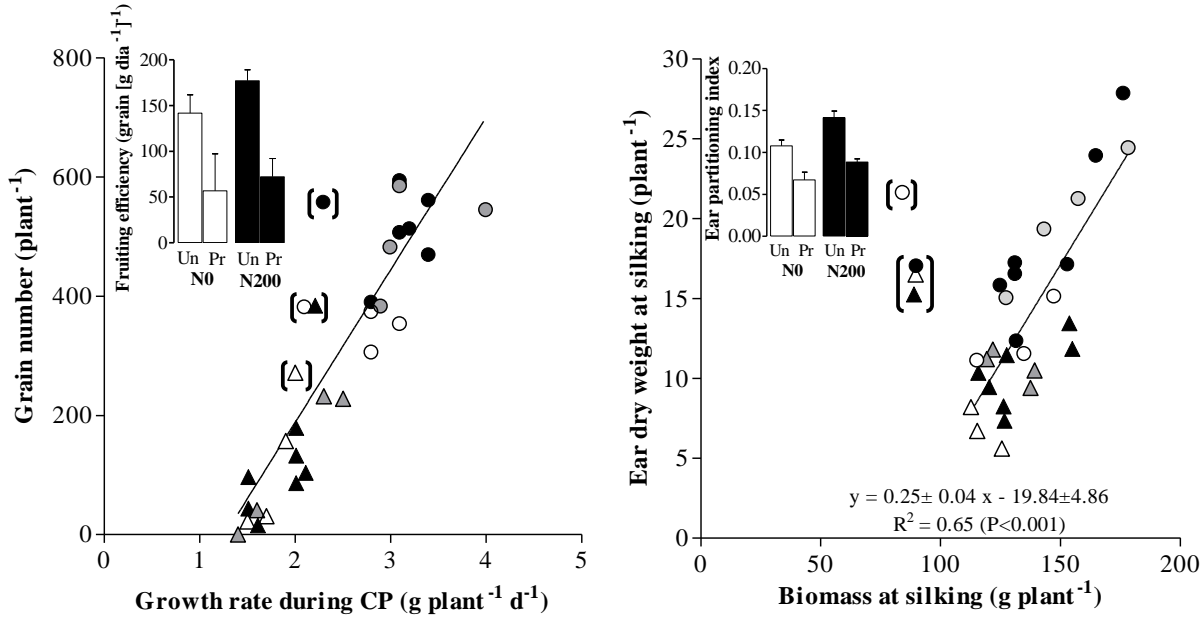
Figure 8. Relationships between the number of grains per plant and the plant growth rate during the critical period (CP) of grain number determination, reflecting differences in the efficiency of conversion of growth during that period into grains set (left panel), and between the ear dry weight at silking and total aboveground biomass at that stage determining differences in the efficiency of partitioning of biomass to the ear (right panel). Data correspond to all high-temperature treatments starting before silking (triangles) and the unheated controls (circles) in factorial combination with different N fertilisation regimes (N0, open symbols; N100, grey symbols, N200, black symbols). Data between square brackets correspond to Lapopi, the short-cycle hybrid also grown in exps 1 and 2. Line is the fitted linear regression not including the data of Lapopi (including them the regression would be Y = 258.4±25.0 x − 341.3±63.5; R^2 = 0.78; P<0.001 in the left panel and Y = 0.09±0.04 x + 2.34±5.14; R^2 = 0.15; P<0.05 in the right panel. Inset on the left panel is the fruiting efficiency (i.e. the efficiency for converting a particular growth rate during the critical period for grain number determination into grains), and on the right panel the ear partitioning index (i.e. the proportion of biomass allocated to ear at silking) averaged for all the cases of the long cycle hybrids with two extreme N doses for the unheated control (Un) and the heat stress starting before silking (Pr); bars stand for the standard error of the means. Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

This implies that a noteworthy part of the effect of heat stress during the critical period for grain determination was indirect: the heat stress affected plant growth and consequently the number of grains (and yield) decreased. However, the reduction in plant growth rate, even when noteworthy, was much smaller than the collapse caused by the treatments in grain number. The intercept of the relationship was significantly negative (Fig. 8, left panel), implying that the intercept on the abscissa, [-a b^{-1}], was significantly positive. This intercept

on the abscissa reflects the minimum plant growth rate during the critical period for allowing grain set to occur was 1.46 g d^{-1} when limiting the regression analysis to the long-cycle hybrids (Fig. 8, left panel; and it was 1.32 g d^{-1} if including the short-cycle hybrid data).

Therefore, the linear relationship reflects a consistent decrease in the efficiency of conversion of growth during this period into grains actually set with the heat stress as well, and the reduction in this sort of "fruiting efficiency" was larger under high- than under low-N availability levels (inset of Fig. 8, left panel). The reduction in fruiting efficiency caused by high-temperature stress starting before silking was even larger than the already noticeable reduction in plant growth rate during the critical period (Fig. 8, left panel). It was due to a dual effect of high temperature stress during the critical period on fruiting efficiency. Firstly, there was another indirect effect through reducing significantly the partitioning of biomass to the ear at silking (Fig. 8, right panel), consequently reducing ear growth during the critical period even more than the reduction in plant growth. Secondly through a direct effect evidenced by the fact that the number of grains set per unit ear dry weight at silking was also dramatically reduced by the heat stress, and more markedly so when the availability of N was larger. The number of grains set per unit ear dry weight at silking under unheated conditions, averaging across experiments and fertilisation regimes, was 26.5 and 28.7 grains g_{ear}^{-1} in N0 and N200, respectively. The corresponding values for the plots being subjected to heat stress starting before silking were 10.0 and 8.5 grains g_{ear}^{-1} in N0 and N200, respectively.

5.3.2 Yield components: average grain weight

The heat treatments imposed during the effective period of grain filling did reduce the average grain weight respect to the unheated controls, whilst when the heat was imposed before silking the average grain weight was not consistently affected (see above, Fig. 7, right panel). The reduction in average grain weight due to post-silking heat stress was only moderate (averaging overall other treatments and experiments grain weight was 286±10.2 and 231±11.5 mg grain^{-1} in treatments unheated and heated during the effective period of grain filling, respectively; representing an overall reduction of 19%). This was likely so, at

least in part, because this treatment did also affect grain number (averaging overall other treatments and experiments there were 493.7±22.8 and 437.7±28.5 grains plant^{-1} in treatments unheated and heated during the effective period of grain filling, respectively). The heat stress during the effective period of grain filling affected more grain weight in the fertilised than in the unfertilised controls, though the effect was not as noticeable as that on grain number by the stress during the critical period. Averaging across other treatments and experiments grain weight was reduced by 40.2±11.0 and 63.4±10.3 mg grain^{-1} representing a reduction of 17.0 and 20.5% under N0 and N200, respectively).

There was a clear relationship between grain weight and plant growth during the effective period of grain filling (Fig. 9, left panel). The relationship was strongly driven by the differences between experiments and N treatments, as the range of biomass accumulation from silking to maturity was similar for unheated and high-temperature treatments imposed 15 d after silking (see ranges in the abscissa of Fig. 9, left panel). Consequently the effect of high-temperature treatments during the effective period of grain growth can be seen in the general pattern of residuals around the regression line, overall (all experiments and N treatments within them) positive for unheated conditions and negative for heat-stressed plants (inset of Fig. 9, left panel).

To determine to what degree these effects were direct (on the capacity of the grains to grow) or indirect (through affecting the source capacity for supporting grain growth), we plotted the final grain weight against a source-sink ratio, estimated as the amount of plant growth after silking per grain set in that plant. In this plot, we fitted the data through a bilinear relationship that assumes that under low source-sink ratios grain weight would be limited by the source strength (and therefore a positive relationship would be expected), whilst at large source-sink ratios grain weight would be largely independent of the source strength and controlled by the capacity of the grains to grow (and then there would be no relationship between grain weight and the source-sink ratio) (Fig. 9, right panel).
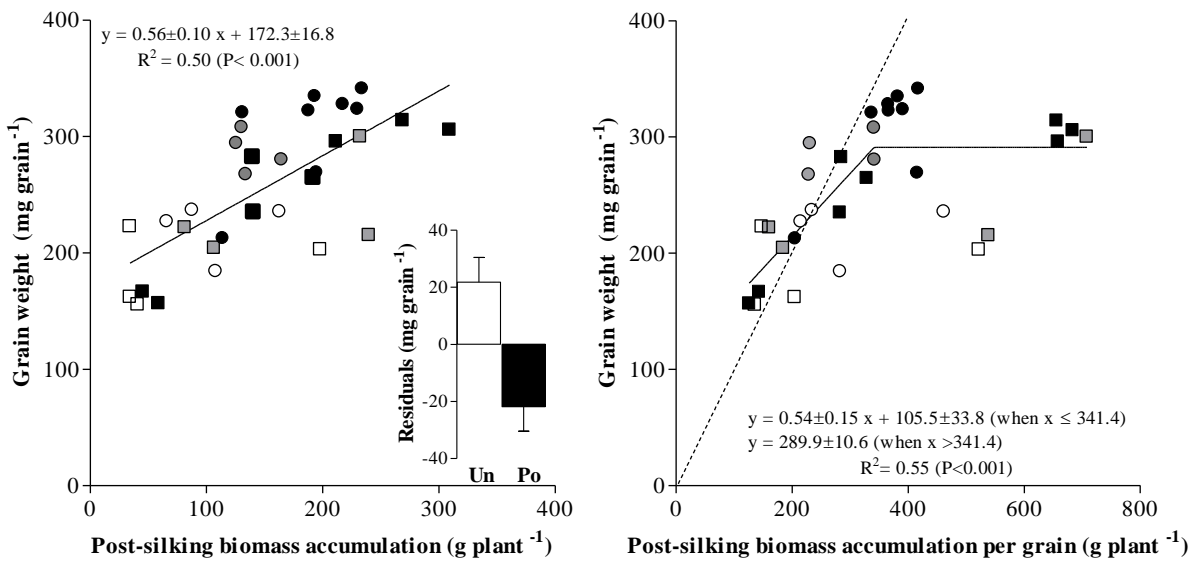
Figure 9. Relationships between the average grain weight and post-silking biomass accumulation either in absolute values (left panel) or relative to the number of grains set in each case (right panel). Data correspond to all high-temperature treatments starting 15 d after silking (squares) and the unheated controls (circles) in a factorial combination with different N fertilisation regimes (N0, open symbols; N100, grey symbols, N200, black symbols). Lines were fitted by linear (left panel) or bi-linear regression (right panel). Inset of the left panel are the residuals under the extreme N fertilisation doses, averaged for all the cases for the unheated control (Un) and the heat stress starting after silking (Po); bars stand for the standard error of the means. Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

In principle, there was not a consistent pattern to allow for a definitive conclusion on the causes for the reduction in grain weight produced by high-temperatures during grain filling. Data-points on the right of the 1:1 ratio are cases in which grain weight would have hardly been source-limited, and within that part of the cloud of data it was clearly consistent that grains exposed to high temperature during the period of grain filling did reach a final weight lower than those under unheated conditions, even though they had much larger source-sink ratios (derived from the reduction of grain number produced by the heat stress imposed 15 d after silking) (Fig. 9, right panel). Data-points on the left of the 1:1 line did show a clear positive relationship between final grain weight and plant growth per grain during post-silking, (Fig. 9, right panel), and were a major contributor to the positive part of the bi-linear relationship fitted to the whole dataset. Within this sub-set (of data-points to the left of the 1:1 ratio) the plants heated from 15 d after silking had lower values of both

variables than the unheated plants, which could be interpreted as a reflection of an indirect effect of temperature on grain growth, through accelerating senescence and source-limiting grain growth more than under unheated conditions. However, there are two reasons for not accepting straightforwardly that heat stress reduced grain weight in this subset through limiting source per grain. Firstly, all these presumably source-limited cases are actually very close to the 1:1 ratio (where it is presumed a sink limitation for grain growth) and chances are that remobilisation of water soluble carbohydrates reserved in vegetative organs before silking would have a potential contribution to grain growth but not to plant growth after silking (as they were part of the pre-silking growth) and therefore the line separating source- and sink-limited grain weight should be biased towards the left by a magnitude equivalent to the amount of water soluble reserves accumulated at silking (a variable that unfortunately we did not measure). Secondly, and supporting the first argument, the actual grain weight of these presumably source-limited cases exhibited the same range of grain weights of the data-points on the right of the 1:1 line (Fig. 9, right panel). Analysing the relationship across the whole range of post-silking biomass accumulation per grain set it is again clear that throughout it (even for the cases on the left of the 1:1 ratio) the residuals of the heated plants were negative (-22.1 mg grain^{-1}) and those of the unheated plants were positive (+5.6 mg grain^{-1}). All in all it seemed most likely that the effects of heat stress during the effective grain filling period directly on the capacity of the grains to grow would have been more relevant than the indirect effects, if any, it may exerted through reducing the source-sink ratio during grain filling.

In fact the imposition of heat stress starting 15 d after silking seemed to have only marginally affected senescence. We did not follow the dynamics of leaf area during grain filling but did measure periodically the chlorophyll content of the ear leaf indirectly through SPAD determinations. The dynamics of chlorophyll content from silking to maturity was best described in most cases by a bi-linear model with a value at silking (the intercept) which is maintained for a certain period until the onset of net chlorophyll loss, from where onwards there is a fairly linear negative relationship determining a certain rate of leaf chlorophyll loss (Table 2).

All these parameters together provide an indication of the senescence process as affected by the treatments and are integrated in the "area under the curve" (AUC) of the SPAD readings over time: the lower the value of the AUC the higher the senescence. The bilinear regressions were in all the 60 cases analysed significant, and highly significant in the majority of the cases (Table 2).

In 56 out of the 60 cases analysed, the regression was actually bi-linear and only in 4 cases it was essentially linear (the onset of chlorophyll loss was at, or before, silking). There was no consistent effect of any treatment producing this lack of bi-linearity.

The most important parameter determining the AUC was the timing of the onset of chlorophyll loss, while the rate of chlorophyll loss was much less relevant in determining the AUC (Table 2, bottom row). In any case there was a clear effect of N availability on the pattern of senescence, and within the heat stress treatments when the stress started before silking there was a clear and consistent reduction in AUC, whilst the stress imposed only during the grain filling period virtually unaffected the pattern of senescence (Table 2; Fig. 10, left panel).

Consequently there was not a solid relationship between grain weight and the AUC: there was a very weak, though statistically significant (P<0.05) positive relation considering all dataset, but it was mainly driven by N treatments and without association within N levels due to high temperature treatments (Fig. 10, right panel). This provides further support to the overall view that grain size reduction imposed by heat stress during grain filling was a direct effect on sink strength rather than an indirect effect mediated through a reduction in source strength.

Table 2. Outputs of the bi-linear relationship between chlorophyll content in the ear leaf, indirectly assessed through SPAD measurements, and thermal time after silking in all the treatments and experiments. The parameters shown are the chlorophyll content at silking (intercept), the onset of chlorophyll loss (the timing after silking when the loss of SPAD units started irreversibly), and the rate of chlorophyll loss from then to maturity. The coefficient of determination (and probability) for each regression is given as well as the area under the curve of chlorophyll content (AUC) over thermal time as an integrated assessment of leaf photosynthetic capacity throughout grain filling. In the last row the coefficient of correlation (R^2) of the linear relationships of the parameters with the resulting AUC are given (with the significance level).

Experiment (growing season)	Treatments			Chlorophyll at silking (SPAD Units)	Onset of chlorophyll loss (ºC d)	Rate of chlorophyll loss(SPAD units [ºC d]-1)	R^2	AUC (SPAD units [ºC d])
	Hybrid	N	Temperature					
Exp. 1 (2009)	Lapopi	N0	Unheated	56.3±1.3	536.7±33.28	-0.126±0.040	0.52 ***	36412
			Post-Silking	52.9±4.0	301.3±129.3	-0.051±0.023	0.34 **	31414
			Pre-Silking	53.9±7.0	0†	-0.024±0.017	0.21 *	31740
		N200	Unheated	58.9±2.3	442.8±117.3	-0.028±0.008	0.23 *	38495
			Post-Silking	57.7±3.6	234.6±285.0	-0.018±0.013	0.24 *	36313
			Pre-Silking	50.8±7.6	364.1±137.3	-0.078±0.043	0.34 **	30281
	PR31N28	N0	Unheated	46.0±3.2	362.6±82.80	-0.066±0.017	0.74 ***	29787
			Post-Silking	49.9±5.1	184.8±179.7	-0.037±0.010	0.46 ***	31793
			Pre-Silking	45.1±3.2	283.4±79.51	-0.061±0.022	0.63 ***	26842
		N200	Unheated	56.4±6.3	338.5±104.9	-0.074±0.027	0.54 ***	36029
			Post-Silking	53.8±3.7	226.1±181.0	-0.036±0.010	0.47 ***	35460
			Pre-Silking	50.8±4.1	431.0±55.10	-0.116±0.022	0.77 ***	31740
Exp. 2 (2010)	Lapopi	N0	Unheated	46.0±6.1	378.9±119.0	-0.091±0.022	0.67 ***	28417
			Post-Silking	41.7±3.0	555.9±51.70	-0.142±0.033	0.83 ***	28707
			Pre-Silking	38.6±4.1	505.8±68.80	-0.107±0.024	0.72 ***	25927
		N200	Unheated	55.0±2.3	535.8±27.30	-0.146±0.020	0.86 ***	39166
			Post-Silking	53.1±5.1	419.9±102.7	-0.098±0.031	0.70 ***	35294
			Pre-Silking	50.4±3.9	510.4±59.00	-0.119±0.023	0.77 ***	35203
	PR31N28	N0	Unheated	49.6±9.3	0†	-0.023±0.013	0.28 *	30585
			Post-Silking	51.0±2.8	0†	-0.041±0.012	0.68 ***	27415
			Pre-Silking	38.9±8.2	0†	-0.034±0.022	0.72 ***	18328
		N200	Unheated	48.2±2.4	540.6±91.80	-0.047±0.014	0.52 ***	37438
			Post-Silking	52.4±3.0	225.5±102.4	-0.053±0.009	0.78 ***	32976
			Pre-Silking	38.1±4.6	0†	-0.028±0.020	0.52 ***	21045

Table 2, continue.

Exp. 3 (2011)	PR31N28	N0	Unheated	42.6±1.8	304.4±45.00	-0.054±0.003	0.91 ***	28408
			Post-Silking	40.8±1.9	376.2±50.60	-0.055±0.004	0.89 ***	30220
			Pre-Silking	32.4±0.9	621.4±49.00	-0.053±0.006	0.87 ***	29619
		N100 4H	Unheated	49.0±1.1	643.1±30.60	-0.103±0.008	0.94 ***	42561
			Post-Silking	43.1±1.5	628.0±49.10	-0.087±0.010	0.87 ***	37817
			Pre-Silking	42.6±0.9	647.3±32.28	-0.084±0.007	0.93 ***	37623
		N100 S-15	Unheated	43.5±1.6	617.4±55.90	-0.086±0.011	0.84 ***	37331
			Post-Silking	41.3±1.5	665.0±44.10	-0.088±0.010	0.86 ***	37238
			Pre-Silking	39.5±1.3	667.2±47.40	-0.078±0.010	0.83 ***	36031
		N200 4H & S-15	Unheated	49.3±1.1	722.0±38.40	-0.118±0.013	0.92 ***	45826
			Post-Silking	48.8±1.5	667.0±45.30	-0.083±0.010	0.85 ***	46907
			Pre-Silking	47.8±1.3	698.9±41.10	-0.078±0.009	0.83 ***	47352
		N200 4H	Unheated	50.8±1.0	551.1±30.00	-0.088±0.005	0.94 ***	41916
			Post-Silking	48.1±1.8	677.4±54.00	-0.086±0.012	0.79 ***	46165
			Pre-Silking	46.5±1.2	646.0±42.90	-0.080±0.008	0.88 ***	43044
		N200 S-15	Unheated	41.5±1.0	682.9±27.80	-0.097±0.007	0.93 ***	36350
			Post-Silking	40.3±0.9	751.7±34.90	-0.095±0.010	0.91 ***	38850
			Pre-Silking	36.0±1.0	704.7±34.00	-0.071±0.007	0.87 ***	33959
Exp. 4 (2012)	PR33Y72	N0	Unheated †	41.2±1.0	363.3±31.20	-0.056±0.004	0.91 ***	29953
			Post-Silking	40.8±3.3	76.10±125.7	-0.030±0.004	0.72 ***	28311
			Pre-Silking	36.9±0.8	467.1 ±52.35	-0.031±0.004	0.76 ***	33557
		N100 4H	Unheated †	50.6±1.4	543.4±51.90	-0.064±0.010	0.71 ***	44944
			Post-Silking	47.0±1.3	617.3±57.70	-0.059±0.012	0.60 ***	44255
			Pre-Silking	46.9±1.1	711.3±28.20	-0.110±0.014	0.78 ***	42760
		N100 S-15	Unheated	51.2±1.9	551.3±64.50	-0.070±0.014	0.59 ***	45219
			Post-Silking	51.9±1.5	539.1±86.80	-0.041±0.011	0.48 ***	49842
			Pre-Silking	48.5±1.0	772.3±33.50	-0.105±0.018	0.67 ***	46923
		N200 4H & S-15	Unheated	52.2±1.0	657.1±35.60	-0.066±0.007	0.79 ***	51850
			Post-Silking	50.3±1.1	518.6±85.80	-0.030±0.006	0.59 ***	51385
			Pre-Silking	47.4±1.3	636.0±39.60	-0.078±0.009	0.79 ***	44503
		N200 4H	Unheated †	53.8±1.1	645.9±30.60	-0.083±0.008	0.85 ***	51594
			Post-Silking	53.7±2.3	150.7±117.2	-0.024±0.003	0.66 ***	49449
			Pre-Silking	47.7±1.1	559.6±33.10	-0.072±0.006	0.87 ***	42623
		N200 S-15	Unheated	52.2±1.5	610.4±45.50	-0.071±0.011	0.80 ***	49113
			Post-Silking	47.1±2.6	705.1±68.50	-0.059±0.012	0.60 ***	48377
			Pre-Silking	45.3±2.2	727.3±34.13	-0.099±0.010	0.84 ***	43561
R² of relationship between parameter and AUC (58				**0.18**; P<0.01	**0.41**; P<0.001	**0.03**; P>0.1		

*, ** and *** mean that the R² was significant at a P<0.05, 0.01, and 0.001, respectively.

† These cases there was a loss in SPAD units from silking onwards (and the relationship was actually linear, not bi-linear)
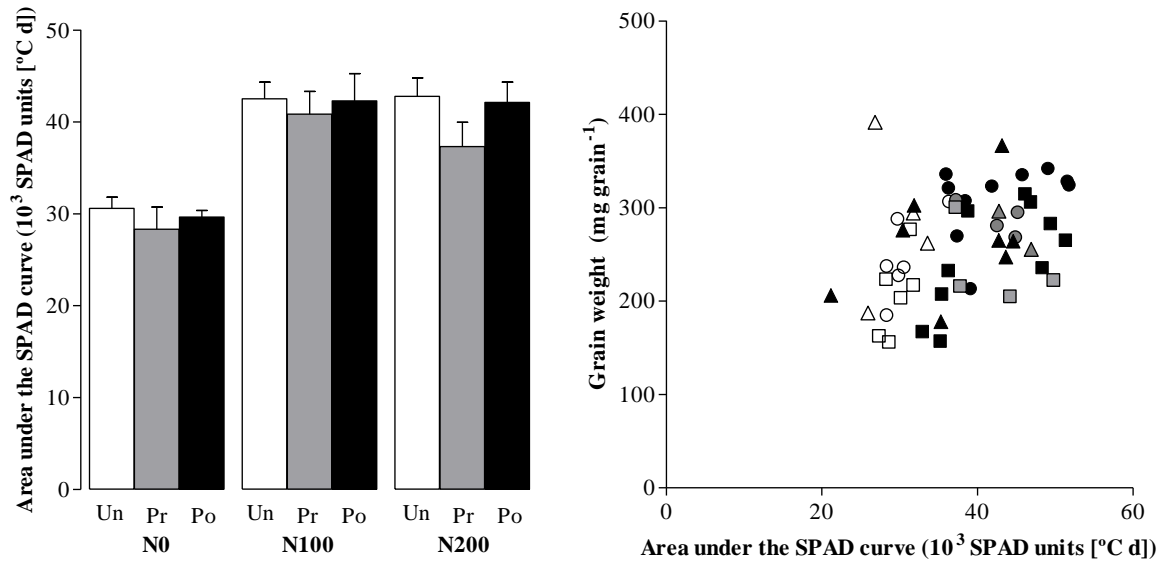
Figure 10. Area under the curve of SPAD measurements over thermal time from silking for the unheated control (open bars) and high-temperature treatments starting before (grey bars) or after silking (black bars) in factorial combination with different N fertilisation regimes (left panel), and relationship between the average weight of the grains and the area under the curve of SPAD for high-temperature treatments starting before (triangles) or after silking (squares) and the unheated controls (circles) in factorial combination with different N fertilisation regimes (N0, open symbols; N100, grey symbols, N200, black symbols) (right panel). Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment)

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5.3.3 Grain protein

The reduction produced by the heat stress on yield was compensated by a concomitant increase in grain protein (Fig. 11, left panel), revealing that even when grain growth might be most likely limited by the sink strength, the protein accumulation in the grain would oppositely limited by the source. Thus, heat stress imposed during the critical period resulted in higher grain protein concentrations than when applied during the effective period of grain filling (Fig. 11, left panel). Overall the data-points felt around the 1:1 ratio between relative changes in yield and protein concentration, as revealed by the fact that the fitted regression had an intercept not significantly different from zero and a slope not significantly different from 1 (Fig. 11, left panel). N fertilisation also favoured grain protein but only when the dose was large, as in N100 the extra growth and yield produced by the fertilisation did not alleviate the source limitation for grain N accumulation compared to the unfertilised controls (Fig. 11, left panel).
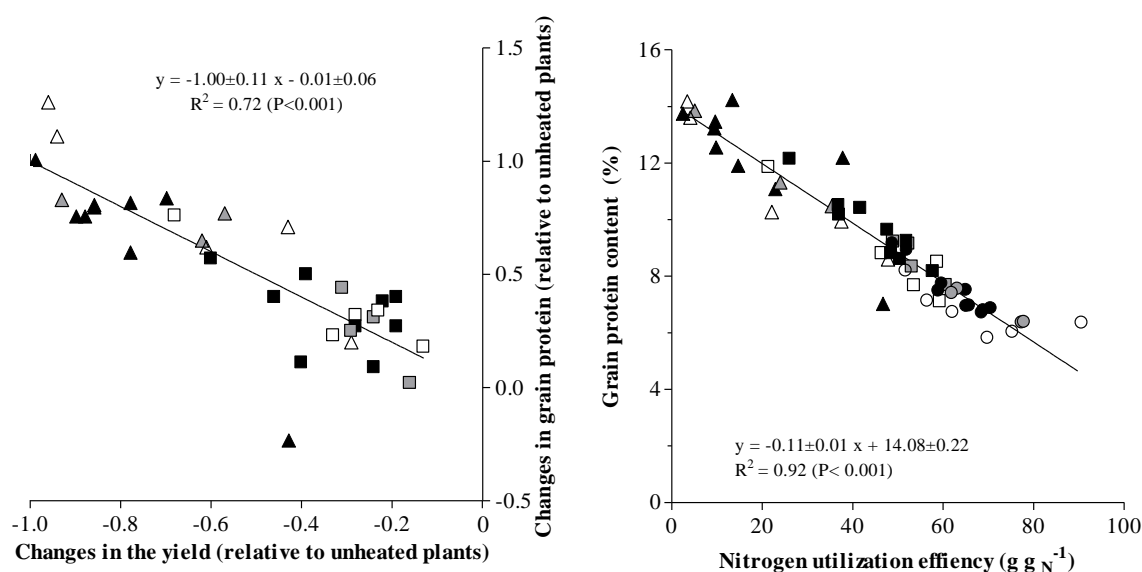
Figure 11. Relationships evidencing the trade-off between yield and grain protein concentration as affected by N availability and heat stress. In the left panel the changes produced by heat stress on yield (decreasing it) and on grain protein concentration (increasing it), estimated as the difference in these variables between the heated and unheated plants relative to the values of the unheated control, are related. In the right panel is the relationship between grain protein concentration and N utilisation efficiency (yield per unit of total N absorbed by the plants during the growing season). In both cases, lines were fitted by linear regression. Data correspond to high-temperature treatments starting before (triangles) or after silking (squares) and the unheated controls (circles) in factorial combination with different N fertilisation regimes (N0, open symbols; N100, grey symbols, N200, black symbols). Each data-point is the average of 9 plants (3 plants per experimental unit and 3 replicated blocks in each experiment).

The basic process seems to be that grain protein concentration seems determined by the degree of dilution of a relatively limited amount of N by the mass of carbohydrates that can be allocated to the grains, mainly limited by the capacity of the grains to accumulate them. As the effects of heat stress, both on number of grains and on grain weight seemed to have operated, at least in a relevant part, through a direct effect on the sink strength (see above), the consequence is the expected trade-off in grain protein concentration. Thus, the higher the N utilisation efficiency (i.e. the higher the yield level per unit of N absorbed by the plants) the lower the grain protein concentration (Fig. 11, right panel). The strongest driving force for this relationship have been the heat stress treatments, though within and across them the effect of N fertilisation can also been seen.

5.4 Discussion

5.4.1 Yield losses due to high temperature x N

In this study we found, in agreement with a large body of literature, that yield of maize (as well as that of other cereals) is highly responsive to N fertilisation (positively) and to heat stress (negatively). However, no studies seem to have been conducted to quantify the likely interaction between these two factors. We found, and as far as we are aware for the first time in maize grown in field conditions, that the losses in yield in response to high temperature were magnified by the availability of N. In other words, that while fertilising with N is rather relevant to maximise yield in most realistic field conditions, it may be also necessary to have into account that well fertilised maize crops may be more sensitive to heat stresses. This may require, more and more often (as the events of heat stress tend to increase with global change), that the doses of N fertilisation be fine-tuned to avoid yield penalties derived from N stress but at the same time to avoid higher yield penalties in the event of heat stress. The results from our study are focused on yield, but the relevance of considering the interaction uncovered would be even more noticeable if we consider the gross margin, considering the costs of the fertilisation.

As mentioned above, these are the first results from field grown maize on the interactions between N fertilisation and heat on maize yield. There are only very limited data available from other cereals. These few results are in agreement with the conclusion we reached in the present study: the yield penalty produced by heat stress was higher under N fertilised treatments than in the unfertilised. This was reported for

wheat (Altenbach et al., 2003; Zahedi et al. 2004; Dupont et al., 2006) as well as for barley (Passarella et al., 2008). The trade-off in grain protein produced by heat stress in the present study is also in line with that reported for wheat by Altenbach et al. (2003) and for barley by Passarella et al. (2008). Therefore, the strength of the conclusions of the present study are no limited to the number of field experiments in which they are based but also in that the limited amount of evidences of this sort of interaction available in the literature for other cereals are all in line with them.

Two further elements of strength in our study are the experimental approach and the magnitude of the treatments. Regarding the approach, all our results come from field experiments, when the vast majority of knowledge on the effects of high temperature is based from extrapolations from more or less controlled conditions. As extrapolation of conclusions from controlled conditions may be difficult to accept straightforwardly (Passioura, 2006), counting with results from field experiments is essential before conclusions could be extrapolated. We are only aware of very few other cases in which high temperature treatments were imposed to maize crops in the field (in all cases from the lab of Prof. Otegui; e.g. Rattalino Edreira et al., 2011 and Cicchino et al., 2010a,b), with an approach similar to that we used in the present study, and that has been used in the past for small-grained cereals (which are far easier to manage due to the size of the plots; e.g. Borghi et al., 1995; Rawson, 1995; Savin et al., 1996; Calderini et al., 1999; Passarella et al., 2002; Ugarte et al., 2007). Regarding the magnitude of treatments, the extrapolation of results can many times be difficult when treatments are too extreme (which is always attractive to researchers as it facilitates detection of effects). N fertilisation treatments were well within normal rates used by farmers in the region (e.g. Berenguer et al., 2009) and temperature increases were not only reasonable in magnitude but also in the way they were imposed: a heating system based on the greenhouse effect allowing a steady increase during de morning and early afternoon followed by a gradual decrease every afternoon-evening to maximum values averaging relatively likely temperatures in realistic hot days of most temperate regions worldwide (daily maximum temperatures, averaging across the canopy, normally below 40 ºC). This is critically important as the effect of heat not only depends on the magnitude of the high temperature used but also on the rate of change in temperatures for imposing the stress (Wahid et al., 2007). The relevance of the rate of increase from minimum to maximum temperatures to have trustworthy conclusions was already evidenced both in

small grained cereals (Savin et al., 1997) and in maize (Crafts-Brandner and Salvucci, 2002). Suddenly imposed treatments, unlike what happens in days with stressful maximum temperatures, do not allow for acclimation processes to take place and therefore might overestimate the magnitude of the penalties actually expected in reality.

Even when the imposed heat stress was relatively mild (maximum temperatures not beyond what can be expected in reality on hot days of temperate areas around silking and grain filling of maize, in most cases only for a few days, and within each day with slow rates of increase) the yield penalty was dramatic when it coincided in full or in part with the critical period for yield determination (c. 30 d bracketing silking). Strengthening the confidence in that our results are not an artefact from our study, they are in line with those observed independently in the other experiments also conducted under field conditions (Fig. 12).

Pooling the data from these previous field studies with our data we can fit single relationships for yield loss in relationship of the yield of the unheated treatment, and that the relationship is much stronger for the heat stress imposed during the critical period than during the effective period of grain growth (Fig. 12, left panel). The magnitude of the absolute loss in yield was lower in the studies conducted by Rattalino Ederira et al. (2012; 2014) and Cicchino et al. (2010b), but that seemed to be only due to the fact that the yield per plant in unheated treatment was also much lower; and therefore the loss of yield as a percentage of the unheated control were rather similarly substantial (inset of Fig. 12, left panel).

The collapse produced by heat stress in the critical period for grain number determination operated exclusively through reducing the number of grain per plant, and again this seems consistent with what was found in the two previous studies under field conditions (Fig. 12, right panel). This means that the large reduction in grain number did not bring about any consistent feed-forward effect: the relatively few grains remaining after the removal of the heat stress did grow normally (average grain weight was similar to that of the unheated plants, and grain protein increased markedly in response to the dramatic increase in source-sink ratio generated by the collapse in grain number).
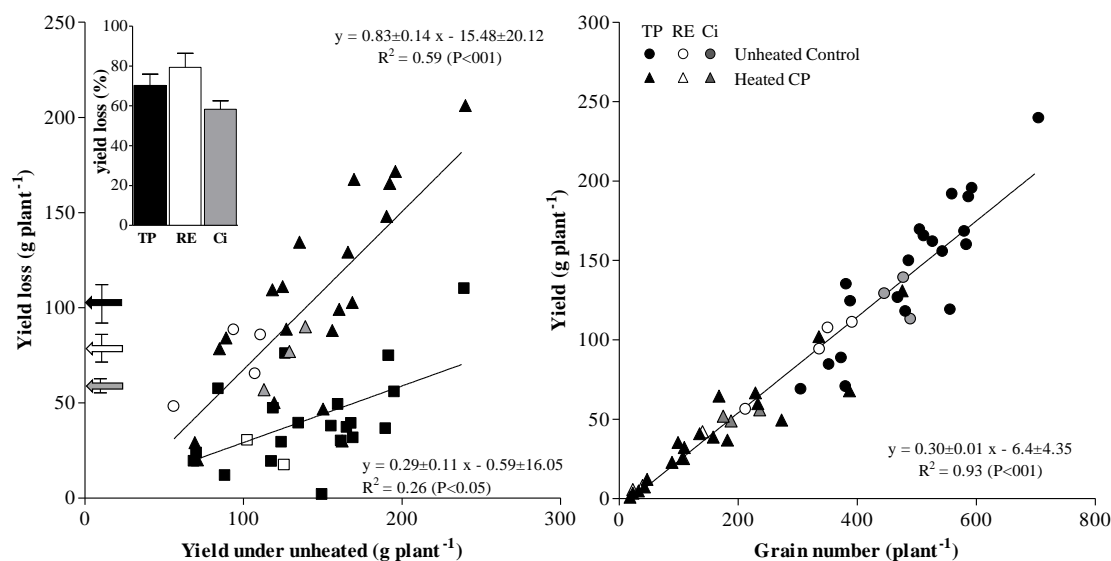
Figure 12. Relationships between yield loss due to heat stress (either during the critical period, triangles; or during the period of effective grain filling, squares) and yield in the unheated control (left panel) and between yield and grain number for the unheated (circles) and heated plants during the critical period (triangles). Closed symbols are data from this paper (TP), open symbols from Rattalino Ederira et al. 2012 and 2014 (RE) and grey symbols from Cicchino et al. 2010b (Ci). Lines fitted by linear regression. Arrows in the left panel stand for the average loss (in absolute terms) for the heat stress imposed during the critical period for grain number determination in each of the studies, and the segment across the arrows is the standard error of that loss. Inset is the same loss but in relative terms (as a percentage of the unheated control).

Although the penalty was less severe than when the stress was imposed during the critical period of grain number determination, heat stress during the effective grain filling period did also affect yield. The effect was through affecting not only the average weight of the growing grains but also the number of grains, although we attempted to impose this treatment after the number of grains has been supposedly fixed (15 d after silking). Again yield was also similarly penalised due to reductions in grain number, in addition to the effects on grain weight, when the heat stress was imposed 15 d after silking in the study by Rattalino Edeira et al. (2014), with the effect slightly stronger in the present study (Fig. 13, left panel).

Naturally the effect of heat stress during grain growth did also affect grain size in both studies in the same direction, though the magnitude of the effect was larger in Rattalino Edeira et al. (2014) than in the present study (Fig. 13, right panel). Again the similitude in the effects reported emphasises the consistency of the results and provide additional support for the trustworthy extrapolation of conclusions. As the experiments by

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Rattalino Edeira et al. (2014) were fertilised with 200 Kg_N ha^{-1}, which was our high-N condition, and N affected positively the magnitude of the penalties imposed by heat stress on both yield components in our study (see above), if we considered only the high-N conditions of our study the effects reported in both studies would be even more similar than what was shown in Figs. 12 and 13 using the overall averages to quantify the effects of heat stress.
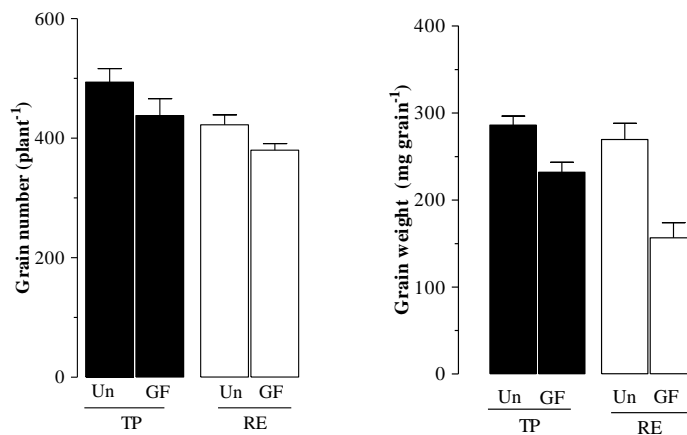
Figure 13. Grain number (left panel) and average grain weight (right panel) in the unheated (Un) and heated plants during the effective of grain filling (GF) in this paper (TP) and in that published by Rattalino Ederira et al. 2014 (RE).

The effect of N on emphasising the penalties seemed not to be a direct effect of this nutrient but and indirect effect through affecting growth. The fact that the results of other independent studies not having N fertilisation as a treatment fit well the same relationship with data of the present study (Fig. 12, left panel) provides further support to the conclusion that the effect of N on affecting the magnitude of the loss was indirect through plant growth, which is in agreement with previous results both in maize (Andrade et al., 2002), and in other cereals (Fischer, 1993; Demotes-Mainard and Jeuffroy, 2004; Prystupa et al., 2004; Ferrante et al., 2010), although exceptions for some genotypes may be found (e.g. D'Andrea et al., 2008).

5.4.2 Crop-physiological bases for temperature x N effects on yield

The extreme yield sensitivity to heat stress during the critical period was not related to changes in phenology caused by high temperatures, as in fact temperature treatments hardly affected time to silking in the present study. It is likely that this lack of effect is revealing that the temperature on the heat-stressed plots would have been above the optimum temperature for at least a significant part of the day. Estimates of relatively low optimum temperatures for the critical period ranging from 30 to 36 ºC were recently reported by Cicchino et al. (2010a) and are in line with optimum temperatures estimated for leaf appearance rates in maize (Kim et al., 2007). Optimum temperatures within the range that plants can be exposed to in realistic field conditions had also been reported for wheat (Slafer and Rawson, 1995). Heat stress does affect the availability and viability of pollen (Schoper et al., 1986; 1987; Mitchell and Petolino, 1988) as pollen desiccation is a function of air temperature and pollen viability decreased linearly with pollen humidity (Aylor, 2003; Fonseca and Westgate, 2005). However, in the present experiments none of the highly expected effects of heat on pollen availability and viability were part of the causes of reduction in grain number when plants were heat-stressed during the critical period as the silks in each of the pants were pollinated with fresh pollen. The fact that yield penalties produced by heat stress were not mitigated by pollinating heated plants with fresh pollen had been already reported (Cicchino et al., 2010b); indicating that the major avenue by which heat affected yield dramatically was indirectly through affecting growth of the ears and/or directly through affecting grain abortion. N fertilisation did not consistently affect phenology either. D'Andrea et al. (2009) compared developmental attributes of inbred lines and hybrids to extremely contrasting N fertilisation regimes (0 and 400 Kg_N ha^{-1}) finding no differences in final leaf number and only relatively marginal advances in silking (averaging 20 and 40 ºC d for lines and hybrids, respectively), with significant genotype x N interactions. This is more or less in line with a lack of consistent evidences from field experiments in other cereals (Hall et al., 2014).

Indeed, it seemed that the collapse in yield produced by the heat stress during the critical period was of such magnitude because of concomitantly occurring direct and indirect effects on grain number loss, and N fertilisation increased the magnitude of both types of effects.

The indirect effects operated through reductions in plant growth during the critical period, which might be reflecting reductions in radiation use efficiency (Cicchino et al., 2010b; Rattalino Edeira and Otegui, 2012); as radiation interception would hardly be affected by relatively short periods of high maximum temperatures starting well after maximum radiation interception has been reached. Important reductions in radiation use efficiency would be expected as high temperatures would concurrently reduce photosynthesis and increase respiration (Penning de Vries et al., 1979; Tollenaar et al., 1989; Crafts-Brandner and Salvucci, 2002; Sinsawat et al., 2004; Hay and Porter, 2006; Kim et al., 2007). This effect on plant growth was complemented with a reduction in partitioning of that growth to the growing juvenile ear. Although the effect might be reversed after the stress is removed (e.g. Cicchino et al., 2010b) abiotic stresses during the critical period of grain number determination do normally reduce dry matter partitioning to the ears (Andrade et al., 2002; Echarte and Tollenaar, 2006; D'Andrea et al., 2008; Suwa et al., 2010).

The direct effects operated through reductions in fruiting efficiency beyond those predictable due to reductions in ear partitioning: heat stressed plants set a much smaller number of grains per unit plant growth during the critical period than the plants which were unheated. No clear mechanisms can be proposed at this stage for these direct effects inducing abortion of pollinated female florets, but the fact that abiotic stresses may induce fertile florets to be abortive, even if grain set is warranted by manipulating pollination, has been already reported (Basetti and Westgate, 1993; Otegui et al., 1995; Cárcova and Otegui, 2001) and possible mechanisms suggested (Barnabás et al., 2008). In the study by Rattalino Edeira et al. (2012), the main damage produced by heat stress during the critical period was also associated with direct more than with indirect effects. These direct effects are largely responsible for the important reduction in harvest index caused by heat stress during the critical period (as the collapse in yield was related to a relatively modest reduction in total growth and N uptake). This effect of heat stress on harvest index is rather common (Ferris et al., 1998; Craufurd et al., 2002; Cicchino et al., 2010b; Rattalino Edeira and Otegui, 2012).

Due to the large direct effects of heat stress during the critical period on grain number through reducing considerably fruiting efficiency, it was expected a likely increase in average grain size due to two physiological mechanisms. Firstly, chances are that abortion of organs follows a hierarchy and therefore grains of smaller size potential

would abort more than those of higher grain potential size (Miralles et al.; 1998, Acreche and Slafer, 2006). Secondly, reductions in fruiting efficiency might be expectedly related to increases in potential size of the grains, as ovaries of the florets might grow more if more assimilates were available per floret reaching the stage of fertile floret (Gambín et al., 2006; Ferrante et al., 2012). In fact, (i) large differences in grain size among crops seems to be based on the ratio between crop growth during the critical period for grain number determination and the number of grains set with those resources (Gambín and Borrás, 2010; Martí and Slafer, 2014); and (ii) if fruiting efficiency is increased by synchronising pollination it promotes parallel decrease in grain weight (Uribelarrea et al., 2008). However, we did not find any consistent increase in grain weight compensating, at least partially, the large reduction in grain number produced by the heat during the critical period. A recent paper also showed evidences supporting that heat stress during the critical period of grain number determination, even when reducing considerably fruiting efficiency, does only marginally affect grain size (Rattalino Edeira et al., 2014). It might be possible then to speculate that there would be a direct effect of temperature on potential size of the grains which is not detectable in the present (and the other above-mentioned) study because it would have been counterweighed by of the potential increase produced by the large improvement in plant growth per grain set.

The lack of any significant feed-forward effect of heat during the critical period on grain growth was also evidences by the fact that, in relative terms, the reductions in yield were quantitatively mirrored by increases in grain protein concentration. Thus the grains set in the plants subjected to heat stress during the grain determination period were not impaired in their capacity to accumulate N, which is normally strongly limited by the source. That is why the effects of the combinations of high temperature x N fertilisation treatments on N utilisation efficiency were very strongly negatively related to grain protein concentration. Similar results were shown for wheat (Pedro et al., 2011; Ferrante et al., 2012) in the absence of heat stress.

When the heat stress was imposed 15 d after silking there was still a significant reduction in grain number, though much more modest than when the heat was imposed in the critical period for grain number determination. This means that it is likely that, at least for the hybrids and background conditions used in the present study and in that carried out by Rattalino Edreira et al. (2012), which were quite different, the critical

period for grain number determination seemed to have actually finished at least a bit later than 15 d after silking. In other words, it is likely that the critical period of grain number determination may have lasted for a bit longer than what is usually acknowledged in the literature. In part it may be difficult to have a universal and incontrovertible duration of this critical period as hybrids may differ in the duration of the lag phase (Maddonni et al., 1998), which is the phase when simultaneously the potential size of the grains is being formed and the final rate of abortion is being determined (i.e. grain number is finally established during the early grain-filling period; Gambín et al., 2008).

Regarding grain weight, although we did not observe any feed-forward effect of heat stress during the critical period of grain number determination, when the plants were heated during the first part of their effective filling period grain weight was significantly reduced (though the magnitude was less than we expected, likely due to the unexpected reduction produced in the number of grains actually set). Again the reduction produced by heat stress on grain weight was larger under high N than under unfertilised conditions.

The analysis of the effect of heat stress treatments during grain filling on biomass accumulation during silking-maturity suggest that the effect on grain weight was not mediated by a limitation of the source strength. The analysis of the effects on the post-silking growth per grain set reinforces the idea that the effects were direct on the capacity of the grains to grow: due to the reduction imposed in grain number, biomass accumulation per grain during post-silking was higher in the heat-stressed than in the unheated plants. Furthermore, the heat stress was mild enough not to allow a clear and consistent acceleration of chlorophyll loss from the leaves. The conclusion that the effect was mainly direct on the capacity of the grains to grow is commensurate with the idea that the effective grain filling is largely sink-limited (Borrás et al., 2004; Gambín et al., 2006; 2008). Rattalino Edeira et al. (2014) have recently concluded that in their study that grain weight reductions due to heat imposed during the early part of the effective grain filling period was not directly related to reductions in assimilate availability per grain but to a direct effect, providing further support to the conclusion from the present study.

The direct effect of temperature may be related to a diminishing enzymatic activity responsible for starch synthesis in the grains (Jenner, 1994). For instance, at high temperature grains could hardly grow even when the concentration of soluble sugars was high (Jones et al., 1981). Thus high temperature effect on grain size cannot be reversed, nor diminished, by increasing the source-sink ratio (Slafer and Miralles, 1992). Another direct effect of heat stress on grain weight might be through increasing the rate of water loss from the grain during the first half of the effective grain filling period (Rattalino Edeira et al., 2014). The dynamics of water content is quite relevant in establishing the final weight of the grains (Borrás et al., 2003; Gambín et al., 2007; Borrás and Gambín, 2010) and treatments affecting this dynamics might impair normal grain growth beyond any effects on availability of soluble sugars to synthesise starch.

We proved for the first time in maize that the sensitivity of yield to heat stress was increased by N fertilisation. This conclusion is based on field experiments with treatments of a magnitude well within expected variation in realistic conditions. The effect was through affecting the capacity of the plants to set grains and to a lesser extent to allow grain weight to be maximised; and it was independent of any (potentially additional) effects on either uncoupling anthesis and silking or on pollen amount and viability.

The influence of N fertilisation on yield was quite universal: it was evident for reductions in grain number (by far the most critical component responsible for the penalties imposed by heat stress) and in average grain weight; and it was clear through both indirect and direct mechanisms of penalising yield components.

5.5 Acknowledgements

5.6 References

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Chapter VI

Maize grain weight sensitivity to source-sink manipulations in a wide range of background environmental conditions

6.1 Introduction

Grain yield is the consequence of the interaction between the number of grains set and their average weight (Slafer, 2003; Borrás and Gambín, 2010). Yield is far better related to grain number than to the average weight of the grains in grain crops in general (Borrás et al., 2004; Slafer et al., 2006), and in maize specifically (Otegui, 1995; Chapman and Edmeades, 1999), because grain number is more plastic (i.e. does respond more to environmental changes) than grain weight (Sadras, 2007; Sadras and Slafer, 2012; Slafer et al., 2014). Even though grain number is the main yield determinant, grain weight is by no means invariable. In fact, relatively large differences in yield can be normally observed for a similar number of grains (as illustrated by Borrás et al., 2004).

It is has been clearly established that variations in grain number are largely related to plant growth during the critical period of grain number determination, from *c*. a couple of weeks before to *c*. a couple of weeks after silking (Edmeades and Daynard, 1979; Kiniry and Ritchie, 1985; Aluko and Fischer, 1988; Tollenaar et al., 1992; Otegui and Bonhomme, 1998; Andrade et al., 1999; Vega et al., 2001; Westgate, 2004; Cicchino et al., 2010; Severini et al., 2011; Rattaliono Edeira and Otegui, 2012).

It is much less clear the physiological causes of grain weight determination. Part of the uncertainties is due to the fact that grain weight potential is firstly set, and then there is a grain weight realisation period. The period of grain weight potential establishment does overlap strongly with the critical period of grain number determination (Calderini et al., 2001; Borrás and Gambín, 2010). During this period (*c*. 20d before to 10 d after anthesis in wheat, Savin and Slafer, 1991; and *c*. 15 d before to 15 d after silking in maize, see above references) it is (i) firstly established the number of florets that will become fertile and pollinated (Kirby, 1988; Otegui, 1997; Cárcova et al., 2003; González et al., 2011) as well as the size of the ovaries of these florets (Calderini et al., 1999), and (ii) secondly the number of endosperm cells which will be later filled with starch and other components are determined, during the "lag phase" between ovule fertilisation and the onset of the effective grain filling period (Brocklehurst, 1977; Reddy and Daynard, 1983; Borrás and Westgate, 2006). It seems that both the size of the ovaries and the number of endosperm cells largely determine the potential size of the grains in relation with the amount of assimilates available per floret/grain during the critical period for grain number determination (Calderini et al., 1999; Gambín et al.,

2006; Ugarte et al., 2007; Borrás and Gambín, 2010; Ferrise et al., 2010; Hasan et al., 2011; Ferrante et al., 2012). Thus the potential size of the grains seems clearly source-limited: the larger the assimilate availability per floret/grain around flowering (before the onset of grain growth) the greater the final size potential of the grain. For instance, if we impose artificial manipulations to reduce fruiting efficiency (the efficiency with which growth around flowering is used to set grains), by lowering the number of florets setting grains but not altering growth and partitioning around flowering, final grain size increases both in wheat (Calderini and Reynolds, 2000) and maize (Gambín et al., 2006). In some cases the reduction in grain size compensates fully the increase in grain number produced by the manipulation (Cárcova et al., 2000).

Final grain weight depends on the potential size established as well as on the realisation of this potential during the effective grain filling period. In wheat and other small grained cereals it is rather clear that in most conditions, grain growth during the effective grain filling period is largely sink-limited (Slafer and Savin, 1994; Richards, 1996; Dreccer et al. 1997; Calderini et al., 2006; Cartelle et al., 2006; Bingham et al., 2007; Pedró et al., 2011; Serrago et al., 2013). In maize the scenario is much less clear. For instance while it is frequently said that in general that potential is realised if the crop does not go through "major limitations" in assimilate availability (Borrás and Westgate, 2006) and then grain growth would be sink-limited during this period (Otegui et al., 1995; Maddonni et al., 1998; Gambín et al., 2008), there are other cases in which the opposite is stated, concluding that there is a clear source-limitation during grain filling (Cerrudo et al., 2013). The controversies may support the statement made long ago by Tollenaar and Daynard (1982) that "a delicate balance exists between sink and source during the grain-filling period of maize and that disturbance of this balance can cause substantial yield reductions". A reflection of that delicate balance was provided by Borrás et al. (2004) when comparing the situation of maize with that of wheat and soybean, following an analysis originally proposed by Slafer and Savin (1994) to determine the degree of source-sink-limitations for grain growth. That analysis showed that while for wheat grain weight does only seldom respond to increases or reductions in assimilate availability per grain during grain filling (Slafer and Savin, 1994; Borrás et al., 2004), in maize grain weight remains largely unchanged when assimilate availability per grain increases but it decreases sharply with reductions in assimilate availability per grain during grain filling (Borrás et al., 2004), leaving undefined whether the most

common situation is a sink- or a source-limitation for the realisation of the potential grain weight.

One difference in the literature is that while in wheat much of what is known on source-sink limitations to grain growth comes from experiments directly manipulating these relationships, in maize most conclusions are based on analyses of the relationship between grain weight and plant growth per grain set during the grain filling period when these variables are modified by various treatments such as sowing dates and densities, N fertilisation regimes (Ahmadi et al., 1993; Maddonni et al., 1998). These treatments affect both sources and sinks and most importantly may also affect kernel weight potential for which it may be difficult to conclude on source- or sink-limitation of grain growth through responsiveness of grain weight when the potential size might have also been affected. Even when some more direct treatments were imposed to alter for instance the number of grains to be filled (through thinning the density or bagging the ears or synchronising pollination; *e.g.* Frey, 1981; Kiniry et al., 1990; Cárcova et al., 2000; Borrás et al., 2001; Sala et al., 2007, though manipulations of source-sink ratios not affecting the number of grains and the potential weight of the grains are also available. Among them, the work of Echarte et al. (2006); Sala et al. (2007) and Severini et al. (2011), defoliating or thinning the plots after the onset of grain growth, is in line with the overall conclusion from the meta-analysis done by Borrás et al. (2004): grain weight was largely unresponsive to increases in resource availability and quite sensitive to reductions in assimilate availability.

In cases in which there were reductions in grain weight in response to defoliations there was an acceleration of leaf senescence as well (Echarte et al., 2006) and then it could be interpreted that the reduction in grain growth could have been due to the accelerated senescence. As crops will be more often exposed to high temperatures (Lobell et al., 2011; Cairns et al., 2013), which would accelerate senescence (Badu Apraku et al., 1983; De la Haba et al., 2014) the effect of high temperatures might be dual: a direct effect on the capacity of the grains to grow (Rattalino Edeira et al., 2014; Ordoñez et al., Chapter V of this thesis) plus an indirect effect through reducing source availability. To the best of our knowledge no experiments have been conducted in maize in which high-temperature treatments had been combined with source-sink manipulations during the effective period of grain filling. In wheat this has been done (Miralles and Slafer, 1992), concluding that the effects of heat stress on grain weight was exclusively direct on the

capacity of the grains to grow. Although the heat stress did accelerate senescence as well, the reduction in grain weight was not reversed by increasing the availability of assimilates per grain (Miralles and Slafer, 1992). As maize source-sink balance is much more delicate than in wheat (Borrás et al., 2004), extrapolations may not be trustworthy and direct experimentation is required.

Another indirect way to estimate whether effects of stresses on grain weight reductions were direct on the capacity of grains to grow or indirect through reducing assimilate availability is studying the response of populations of grains of different potential sizes. In maize grains of the tip of the ear are constitutively smaller than those of the basal and central thirds of the ear (*e.g.* Tolleenaar and Daynard, 1978; Chen et al., 2013). To the best of our knowledge this has not be routinely done in most of the many experiments in which source-sink relationships had been manipulated to determine whether grains are source- or sink-limited during the effective period of grain filling. The paper by Andrade and Ferreiro (1996) seems the exception in which the effect of shading or thinning after the lag phase was measured in grains of the tip and of the base of the ears.

A treatment that has been very popular in wheat and barley studies is the removal of half of the population of grains, after the potential size of the grains has been fixed (Slafer and Savin, 1994; Miralles et al., 1995; Calderini et al., 2006; Serrago et al., 2013). This is a clean way to observe how the remaining grains grow having halved the potential competition compared with the control; if in that case grains were limited by the source. In maize this is far more difficult, as the grains are not exposed, and to the best of our knowledge this sort of treatment has never been applied before to analyse the response of grains to a strongly reduced competition. However, it has been recently reported that applying this sort of treatment might be feasible. Gambin et al., (2007) did apply a treatment eliminating few grains from adjacent rows 15 d after silking to avoid effects on grain size potential to test whether the final size of grains in maize might be limited by lack to space to expand, and for that reason they purposely avoided modifying the source-sink balances. But illustrated how the treatments might be imposed in maize and proved the approach was feasible.

In this paper we report on the responsiveness of grain weight to defoliation and degraining treatments imposed 15 d after silking to maize plants grown under a wide

range of conditions including the imposition of heat stress in combination with N fertilisation regimes.

6.2 Materials and Methods

6.2.1 General and background conditions

Seven field experiments were conducted on actual farmer's paddocks (rented for the experiments to be established using the normal sowing and management of the farmer, with the exception of the N fertilization). All experiments were in the province of Lleida (Catalonia, north-eastern Spain). In the first two growing seasons (2009 and 2010) experiments were conducted in two sites differing in altitude: close to Algerri (41º47´41´´ N; 0º38´52´´ E; 230 m alt), a relatively warm location within the Plain of the province and part of the valley of the Ebro River (two sowing dates in the second year); and close to La Seu d`Urgell (42º 20' 45" N; 1º 25' 52" E; 730 m alt), a relatively cool Valley in the middle of the Pyrenees (experiments 1-5). In the second of these seasons in Algerri there were two experiments sown with 1 month difference. In the last two growing seasons (2011 and 2012) experiments were conducted only in the Plain of Lleida, again close to Algerri (experiments 6-7) (Table 1).

All experiments were sown within the normal sowing dates for the region within each location and at a plant density within the range considered optimum (Table 1). The seven experiments were maintained free of water stress through periodic irrigations with sprinklers (exps. 1, 2, and 5) or drip irrigation systems (exps. 4, 6 and 7) maintaining soil moisture close to field capacity throughout the growing season. Weeds, pest and diseases were prevented or controlled by spraying recommended herbicides, insecticides and fungicides at the doses suggested by their manufacturers whenever necessary.

The experiments were sown with 8-row sowing machines (0.70 or 0.75 m apart in Algerri and La Seu d'Urgell, respectively) and the main plots in each site were the width of a sowing machine and 20-40 m long, depending on the experiment (i.e. in all cases main plots were large). There were two distinct hybrids in the first five experiments and only one in the last two (in which the number of N fertilisation regimes increased) (Table 1).

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Table 1. Year, number of experiment, soil characteristics, sowing date and density of the seven field experiments and treatments in each of them that comprise the background characteristics in which the source-sink treatments were imposed.

Year	Experiment number	Location (fields were close to)	Soil characteristics			Sowing date	Plant density (plants m^{-2})	N fertilisation	Hybrid	Heat stress
			OM (%)	N-NO$_3$ (kg ha^{-1})	P (kg ha^{-1})					
2009	*Exp. 1*	Algerri (Plain of Lleida)	1.9*	175***	70,0*	12 May	8.03	N0 N200$_{6H†}$	Lapopi PR31N28	Unheated Heat$_{15DAS-Mat}$‡
2009	*Exp. 2*	Seu d`Urgell (Pyrenees)	1.4*	150**	88,2*	11 May	8.50	N0 N200$_{6H†}$	Lapopi PR31N28	Unheated
2010	*Exp. 3*	Algerri (Plain of Lleida)	1.5*	141***	35.1*	16 April	8.40	N0 N200$_{6H†}$	Lapopi PR31N28	Unheated Heat$_{15DAS-Mat}$
2010	*Exp. 4*	Algerri (Plain of Lleida)	1.5*	141***	35.1*	17 May	8.40	N0 N200$_{6H†}$	Lapopi PR31N28	Unheated
2010	*Exp. 5*	Seu d`Urgell (Pyrenees)	1.4*	129**	70.0*	20 May	8.50	N0 N200$_{6H†}$	Lapopi PR31N28	Unheated

Continue table 1.

2011	*Exp. 6*	Algerri (Plain of Lleida)	1.5*	225***	72.1*	31 March	8.40	N0 N100$_{4H\dagger}$ N100$_{S-15\dagger}$ N200$_{4H}$ N200$_{S-15}$ N200$_{4H+S-15}$	PR31N28	Unheated Heat$_{14DAS-32DAS}$
2012	*Exp. 7*	Algerri (Plain of Lleida)	1.5*	177***	9.7*	20 March	8.40	N0 N100$_{4H}$ N100$_{S-15}$ N200$_{4H}$ N200$_{S-15}$ N200$_{4H+S-15}$	PR33Y72	Unheated Heat$_{14DAS-32DAS}$

*Top 0.30 m of soil depth; ** top 0.75 m of soil depth; ***top 1 m of soil depth
†4H, 6H and S-15 stand for V4, V6 and 15 d before silking, respectively. 4H+ S-15 means half was applied in V4 and the other half 15 d before silking.
‡DAS stand for days after silking; Mat for maturity

The hybrids grown were Lapopi (included in exps. 1-5) classified as FAO 450, and Pioneer 31N28 (PR31N28 included in exps. 1-6) or Pioneer 33Y72 (PR33Y72 included in exp. 7), both classified as FAO 700 (Table 1), selected for representing single hybrids of different cycle duration but well adapted to the region.

On these basic frameworks we included different environmental treatments. They were different N fertilisation regimes in the seven experiments and two heat stress conditions in four of the experiments: a control unheated and a heat stress imposed during the effective period of grain filling (exps. 1 and 3) or during the first 15 d of it (exps. 6 and 7) (Table 1). All in all (experiments x hybrids x N regimes x heat treatments) we had a rather varied arrangement of 52 background conditions in which we imposed the source-sink manipulations. For details on the imposition of these background treatments and on experimental design, please see Chapters III and V. In all the seven experiments there were three replicates arranged in blocks.

6.2.2 Source-sink manipulations

In the present study, manipulation of source-sink relationships were restricted to the timing of the effective grain filling period as the imposition of the treatments was done in each case 15 d after silking. Therefore, we do not expect any confounding effect of manipulations hypothetically altering grain size potential in addition to the expected changes in assimilate availability per grain. For this purpose plants were selected for being at the exact planting density and with uniform distribution with their border plants and labelled within each particular background condition: in exps. 1-5 six plants were labelled, in exps. 6-7 ten plants were labelled. All these plants selected were identical at naked eye (equal size, leaf number and stage of development) within experimental unit in which the manipulations were imposed as sub-plots. These experimental units, in which source-sink manipulations were imposed, were the combination of experiment x hybrid x N regime x heat stress condition. And the imposition of treatments was 15 d after silking in each of these units (*i.e*. different for different plots within the same block, depending on how the background conditions may have affected time of silking). Within these sub-plots each source-sink manipulation (control, defoliated and degrained) was performed on three

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different plants (and in the experiments with degraining treatments there was an additional plant used as a placebo, as explained below). As there were three replicated blocks in each of the experiments, all in all there were 9 plants for each source sink manipulation within each of the background conditions in which they were imposed. In all cases (the 52 different background conditions across all the seven experiments, Table 1) we had a control not being manipulated as well as a potential source-restriction through defoliations, and in 12 of these background conditions (exps. 6 and 7) we also imposed a treatment dramatically increasing the potential availability of assimilates for each growing grain during the effective period of grain growth through a novel degraining treatment.

Defoliations were made simply by removing leaf laminae from most leaf positions. The leaf removal was done by cutting the leaves on the collar, between the leaf lamina and leaf sheath. In exps. 1 and 2 we removed all the leaf laminae but two: the two leaves adjacent to the ear (those immediately below and above) were left untouched and all the others removed. In exps. 3-7 we removed two less leaf laminae tan in the first growing season: the third leaf below the ear, the leaf adjacent to the ear, the 3rd leaf above the ear and the flag leaf were left untouched and all the others removed. These defoliations represented c. 75 and 65% reduction in total leaf area at the timing of treatment imposition in exps. 1-2 and 3-7, respectively.

Degraining was performed in exps. 6 and 7 through a novel approach, provoking alternate rows of grains of the ear to die (Fig. 1). The procedure started with carefully opening the husks pulling them back from the tip to the base (like peeling a banana), and removing the mature brownish silks. Then the opened ear was sprayed with 96º alcohol and with a scalpel previously disinfected all grains within a row of alternate rows were damaged, roughly halving the number of grains. After performing the treatment, a broad spectrum fungicide (Chlorothalonil 50% w/v suspension concentrate) was sprayed to the ears and finally the husks were returned as close as possible to their original position and maintained so with loose elastic band on the tip. In addition, to the three plants manipulated in each of the 36 experimental units (12 background N x temperature conditions and 3 replicates), in the 18 unheated ones we also had a sort of placebo to determine whether the manipulation to produce the degraining might have affected grain growth by itself. In these plants (1 per

rep, 3 per N regime treatment under unheated conditions) we did all the same procedure but not using the scalpel (we peeled back the husks of the ear, sprayed firstly with alcohol and then with the fungicide, and finally closed the husks and kept them together with a loosely fitted elastic band on the tip).

6.2.3 Sampling and determinations

At physiological maturity all plants treated were sampled and taken to the lab. In the lab the ears of each plant were divided in three sections: the basal, central and apical thirds of each ear. In each third we separate the grains from the cob and counted them. Then we oven-dried the grains for 72 h at 65 ºC and weighed them. We then joined the grains from the different thirds, milled the samples and determined N content by Kjeldahl.

Figure 1. *Top panel*: illustration of some steps of the procedure to perform the degraining treatments, firstly carefully opening the husks (left), then –after spraying the ear with alcohol-removing all grains in rows of alternate rows with a disinfected scalpel (middle), and finally -after spraying the ear with fungicide- closing back the husks and keep them close to the original situation with a loose elastic band (right). *Bottom panel*: images showing ears 15 d after silking without and with the degraining imposed (left) and ears without and with the degraining in maturity (right).

6.3 Results

The combinations of experiments x hybrids x N regimes x heat treatments produced 52 background conditions in which grain number ranged from less than 200 to more than 700 grains plant^{-1} and the average grain weight ranged from c. 150 to c. 350 mg grain^{-1} (Fig. 2).
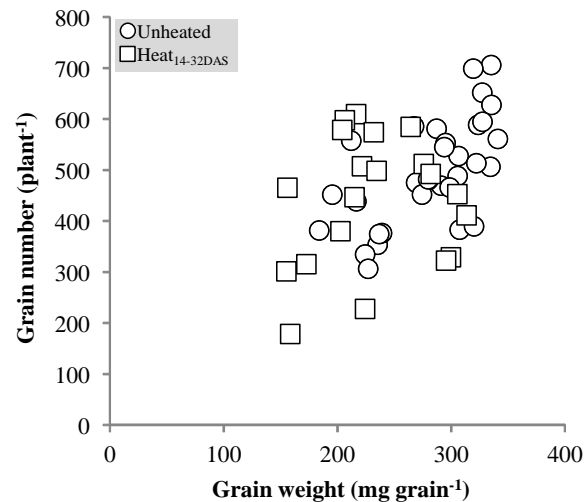
Figure 2. Ranges of variation in grains per plant and in average grain weight produced by the combinations of experiments x hybrids x N regimes x heat treatments (Table 1) on which the source-sink manipulations were imposed. In the figure data-points belonging to the unheated controls (circles) and to the plots heat-stressed during the first 17 d of the effective period of grain filling (squares) are identified.

The heat stress imposed at the onset of the effective grain filling period reduced both grain number and average grain weight and the ranges within the unheated and heated in grain filling were due to the different N fertilisation regimes and the different experiments. Within each of these two thermal regimes there was no relationship between grain number per plant and average grain weight (Fig. 2).

This means that the source-sink treatments were imposed to wide combination of grain number and average grain weight in the controls not defoliated nor degrained, which was an expected feature of the study.

6.3.1 Responses to defoliation

Defoliation drastically reduced the average weight of the grains in 6 out of the 7 experiments in which the treatment was imposed (Fig. 3). Within these 6 experiments there were also heat stress treatments during grain filling in 3 of them, which in turn reduced the weight of the grains as well. But the effect of defoliation was not stronger in heated than in unheated plants (Fig. 3), as it would be expected if the reason for the decrease in grain weight due to heat stress would have been the reduction in assimilate availability associated with the increased temperature.

Exp. 7 was the exception in that it did not exhibit a reduction in average grain weight when plants were defoliated (squares, Fig. 3) In this experiment high temperatures during grain filling did reduce grain weight as well, but defoliation in this case did not reduce the final weight of the grains of plants exposed to heat-stressed conditions either, reinforcing the hypothesis that the effect of heat stress was not mediated through reductions in assimilate availability.

Analysing responsiveness of grain weight to defoliation across all experiments together, there seemed to be a single relationship for all cases, excluding exp. 7, between the magnitude of the effect of defoliation and the weight of the grains in the control not defoliated plants (Fig. 4). The relationship broadly indicates that when the conditions (environmental conditions given by different experiments and different N fertilisation regimes within experiments) lead to larger grains, these grains might be more sensitive to defoliation than when the crop sets smaller grains (Fig. 4). This general argument does not hold up when the condition reducing grain weight is the exposure to heat stress during grain filling. Thus, even when in the control these grains are clearly smaller than in the unheated plants the reduction imposed by defoliation was similar (Fig. 3).
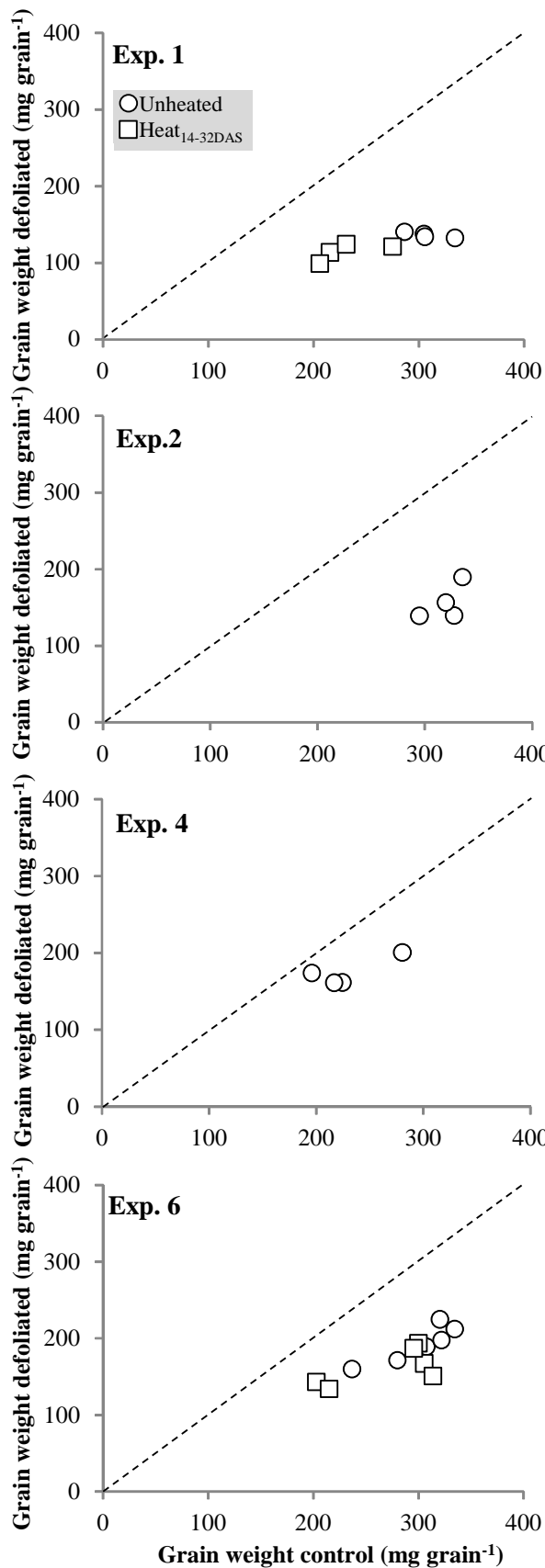
Figure 3. Grain weight averaged for the whole ear in the defoliated plants plotted against the values corresponding to the control not defoliated in each of the 7 experiments under unheated (circles) or heated during the effective period of grain filling (squares). Dashed lines represent y = x, the 1:1 ratio.

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Consequently the residuals of the data-points corresponding to the heat stressed plants were significantly positive and those belonging to the unheated plants were significantly negative (Fig. 4, right panel). This does not mean that the defoliation produce stronger penalties in heat-stressed plants, as it might be linearly interpreted; it simply reflects that the reduction in grain size produced by heat stress did not change the sensitivity to defoliation.
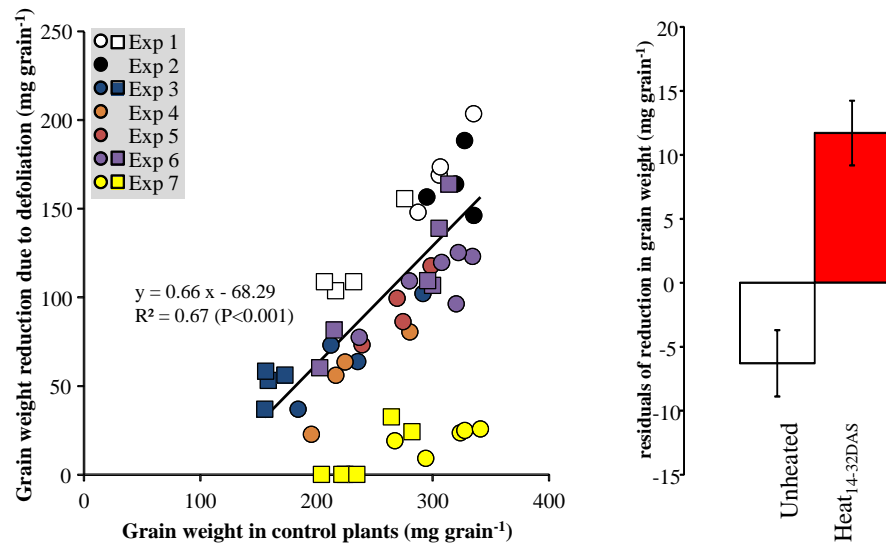
Figure 4. Relationship between the reduction in grain weight produced by the defoliation and the weight of the grains in the not defoliated controls under unheated (circles) or heated during grain filling (squares) in the seven experiment with line fitted by linear regression, excluding data-points of exp 7 (left panel); and residuals respect to the regression line average for the heat stressed and unheated plants (segments stand for the standard error of the means).

When analysing the response of different populations of grains (those of the base, the middle or the tip of the ear, which are constitutively different in size) we surprisingly found that overall there were no clear differences in responsiveness to defoliation (Fig. 5). Heat stress did not consistently increase the damage in the weakest grains (Fig. 5), and this supports the idea that heat stress does affect grain weight directly and not through increased restrictions of assimilate availability. On the other hand, it was unexpected that defoliation did not consistently penalised more the weight of the grains in the apical third of the ear (Fig. 5), as it would be expected that a reduction in assimilate availability would have affected more the weakest grains.
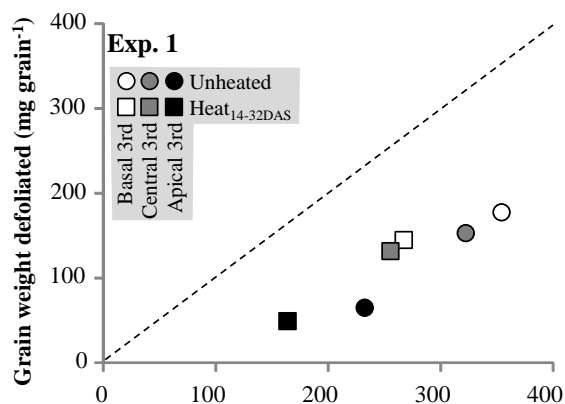
Figure 5. Grain weight of the basal, central and apical thirds of the ears (open, grey and closed symbols, respectively) in the defoliated plants plotted against the values corresponding to the control not defoliated in each of the 7 experiments under heat stress (squares) and unheated conditions (circles). Each data-point is the average of all N fertilisation x hybrids treatments within each experiment. Dashed lines represent y = x, the 1:1 ratio.

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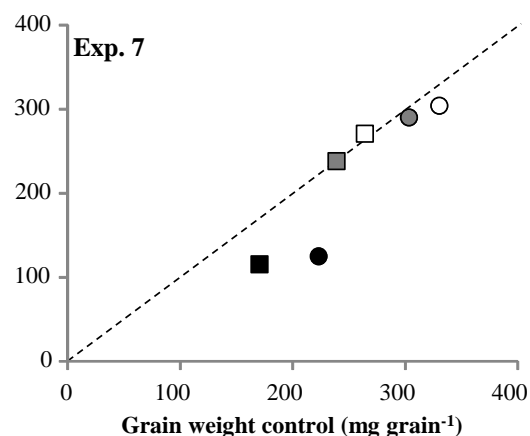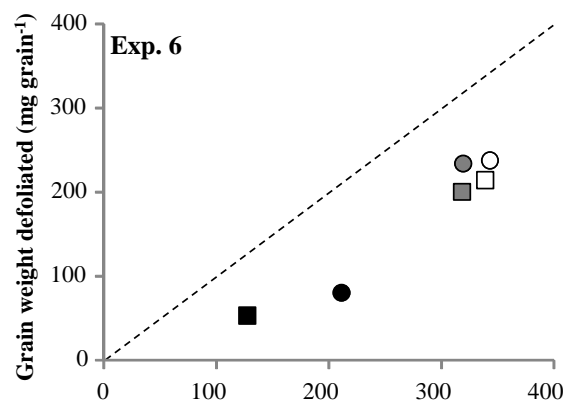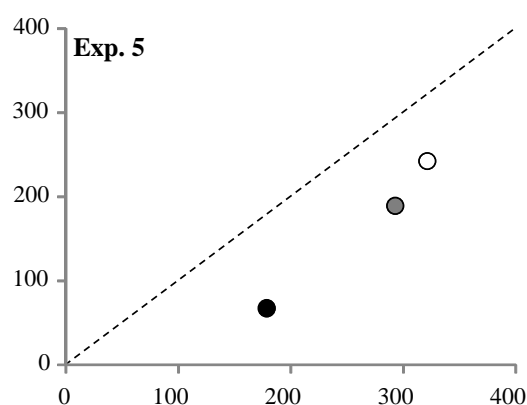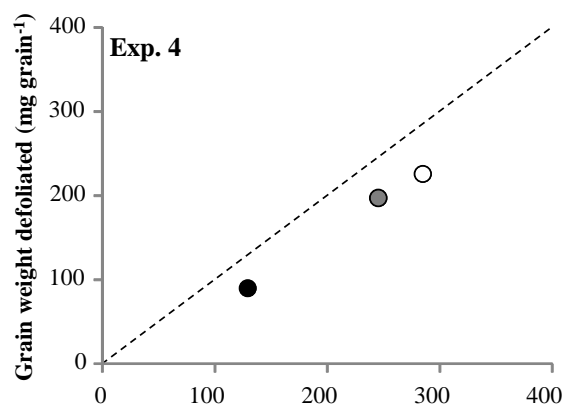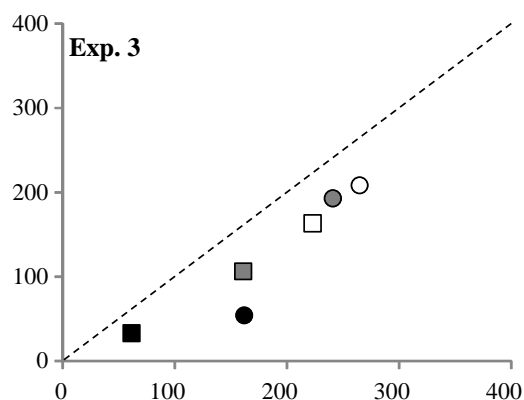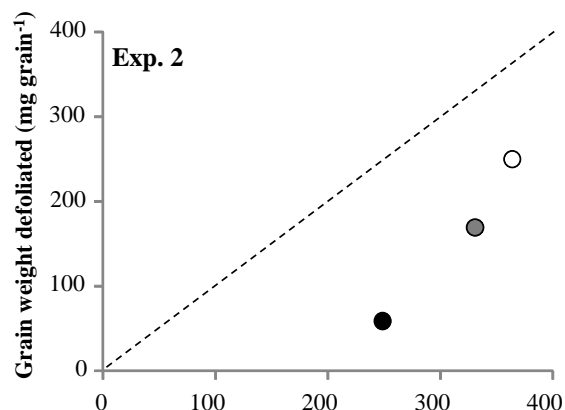
Defoliation in general tended to increase grain N concentration, though the increase was less than expected from reductions in grain size (Fig. 6) likely because the same treatment that reduced grain weight did also reduce the source of N for grain filling.
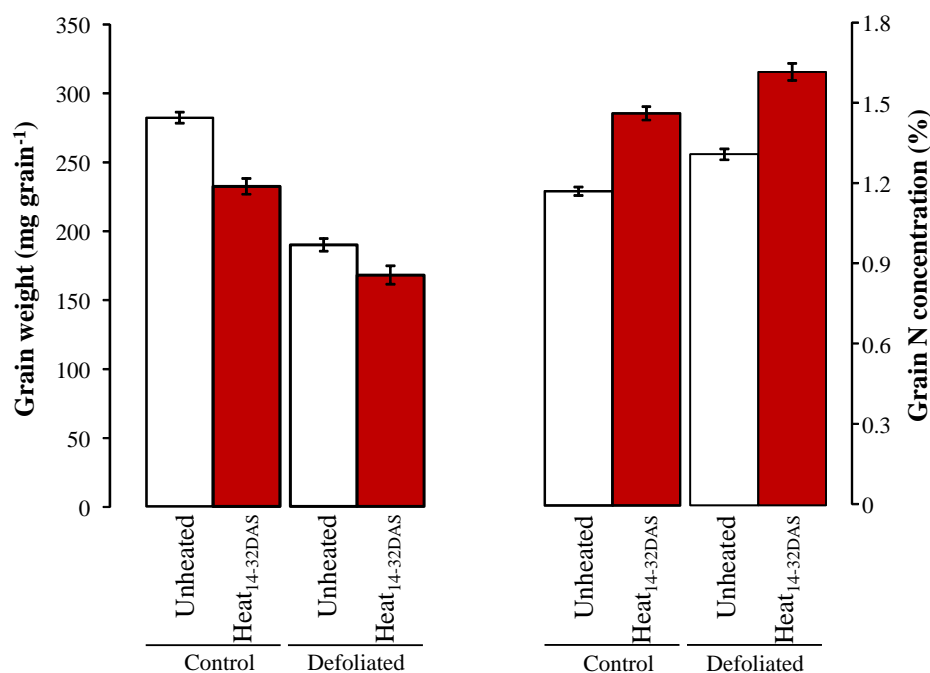
Figure 6. Average weight (left) and grain N concentration (right) of grains from plants which were either unheated or heat stressed during the effective period of grain filling in factorial combination with two different source-sink balances: control, not defoliated, plants and defoliated (65-75%) 15 d after silking. Segments on each bar stand for the standard error of the means.

Thus, whilst the increase in protein concentration due to heat stress was proportional to the decrease produced in grain size, when the reduction in grain weight was produced by defoliation the increase in protein concentration was relatively marginal (Fig. 6). In fact, the grains of the plants defoliated but unheated were in average smaller than those from plants not defoliated but exposed to heat stress during grain filling, but their grain protein concentration was also lower (Fig. 6).

6.3.2 Responses to degraining

We imposed a novel approach to determine to what degree grains compete for limited resources during the effective period of grain filling, once their potential size has been established. The first doubt issue to resolve was whether the approach was minimally valid: whether the magnitude of the treatment was similar to that expected and whether it might have unintentionally affected the growth of the remaining grains. The latter issue constitutes a major potential inconvenience of the approach as it is necessarily quite intrusive. To test the appropriateness of the method, we established a number of treatments fulfilling the role of what would be placebos in pharmacology studies: we did all the manipulations required to perform the degraining but without provoking any abortion and determined effects in final grain weight compared to the control without manipulation.

Firstly, the treatment did effectively reduce the number of grain within the expected range (Fig. 7, left panel). Grain number in the degrained plants were in average slightly less than half the number of grains in the control plants, under either heat stress condition (Fig. 7, left panel). In the control plants which were not manipulated grain number ranged from *c*. 300 to *c*. 600 grains plant^{-1} in unheated conditions and from *c*. 250 to *c*. 550 grains plant^{-1} in plants exposed to heat stress 15 d after silking. In the degrained plants these ranges were *c*. 125-300 and 100-300 grains plant^{-1}, respectively.

Secondly, we found no consistent effects of the manipulation required for the degraining on the final size of the grains (Fig. 7, right panel). There was some variation around the 1:1 ratio between the weight of the grains in the placebos and the not manipulated controls which is expected as even when the plants selected for applying the treatments (including the placebo) were selected to have identical appearance they cannot expected to be strictly identical. As we had a limited number of plants for the placebos, the error in their average was larger than in the not manipulated controls, for which we had three fold more plants to average (Fig. 7). All in all it seems that the manipulation required to impose the treatment did not produce any significant impairment on the capacity of the grains to grow similarly to those of the not manipulated control plants.
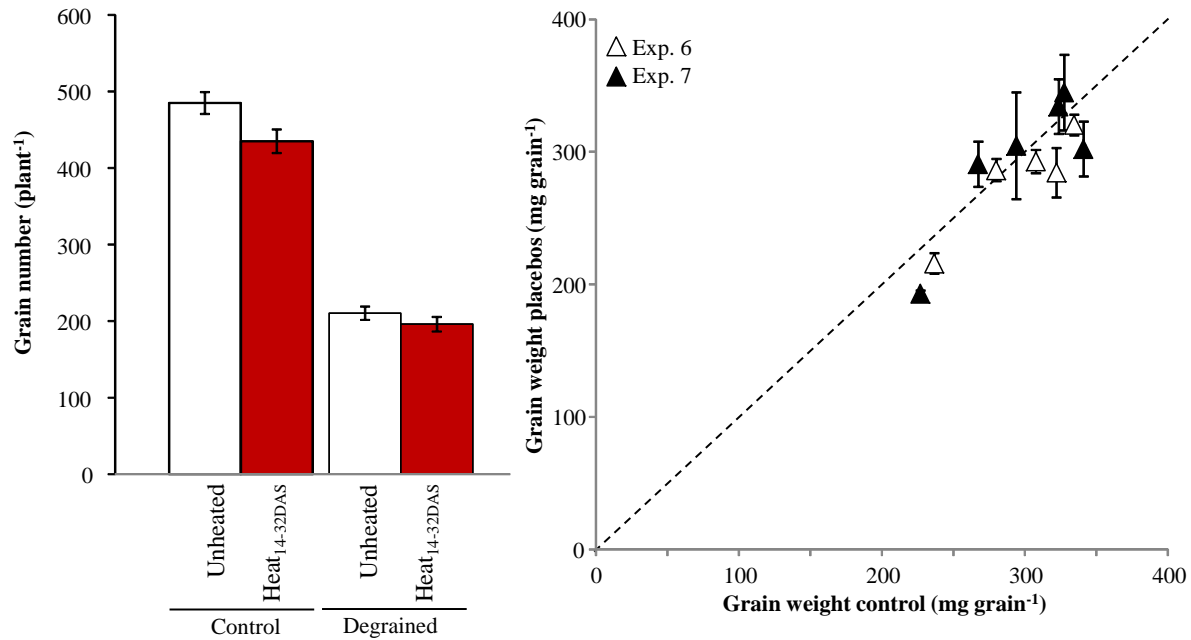
Figure 7. *Left panel*: number of grains per plant in the not manipulated control plants and in the plants subjected to the degraining treatments under unheated and heat-stressed conditions averaged across N fertilisation regimes and experiments. Segments stand for the standard error of the means. *Right panel*: Grain weight of the of the plants subjected to the whole manipulation required for the degraining but without degraining (placebo) plotted against the weight of the grains in the controls not manipulated at all in exps. 6 (open symbols) and 7 (closed symbols). Each data-point is the average of 3 plants for the placebos and of 9 plants on the not manipulated control and different data-points within experiments belong to the different N fertilisation regimes. Segments on each symbol stand for the standard error of the means. Dashed line represents y = x, the 1:1 ratio.

The degraining treatment did not increase grain weight consistently in any of the experiments, neither under unheated nor under heat-stressed conditions (Fig. 8). Although there seemed to be a trend for the grain weight of the heat-stressed plants to be more responsive the differences were not significant. Grain weight in the degrained plants was not increased at all under unheated conditions and the increase in the heat-stressed plants was negligible compared with the reduction provoked in grain number.
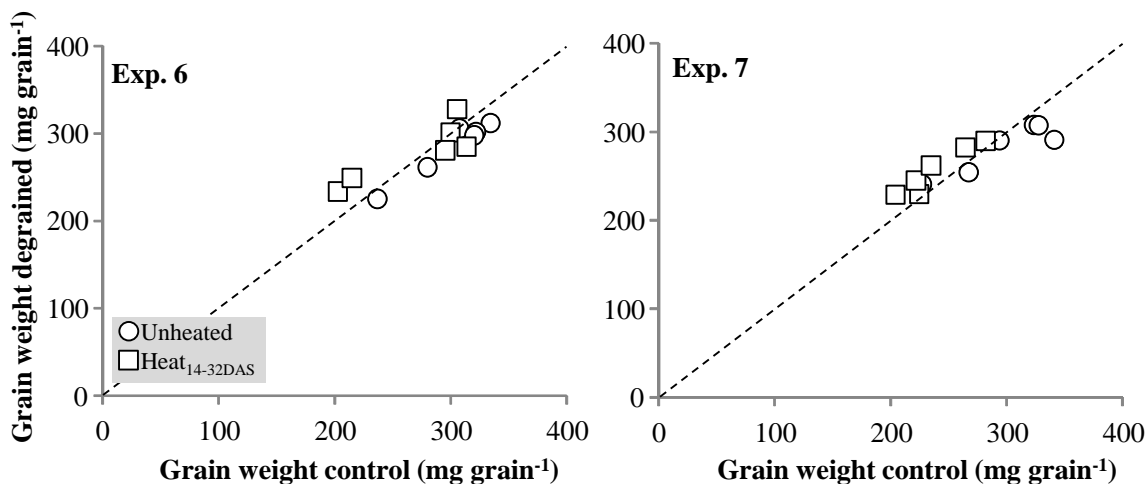
Figure 8. Grain weight averaged for the whole ear in the degrained plants plotted against the values corresponding to the control not defoliated in each of the 2 experiments in which this treatment was imposed under unheated (circles) or heated during the effective period of grain filling (squares). Dashed lines represent y = x, the 1:1 ratio.

Again the lack of clear and consistent increases in grain weight in response to degraining was not only true for the whole population of grains in each condition but also for grains from particular thirds of the ears (Fig. 9).
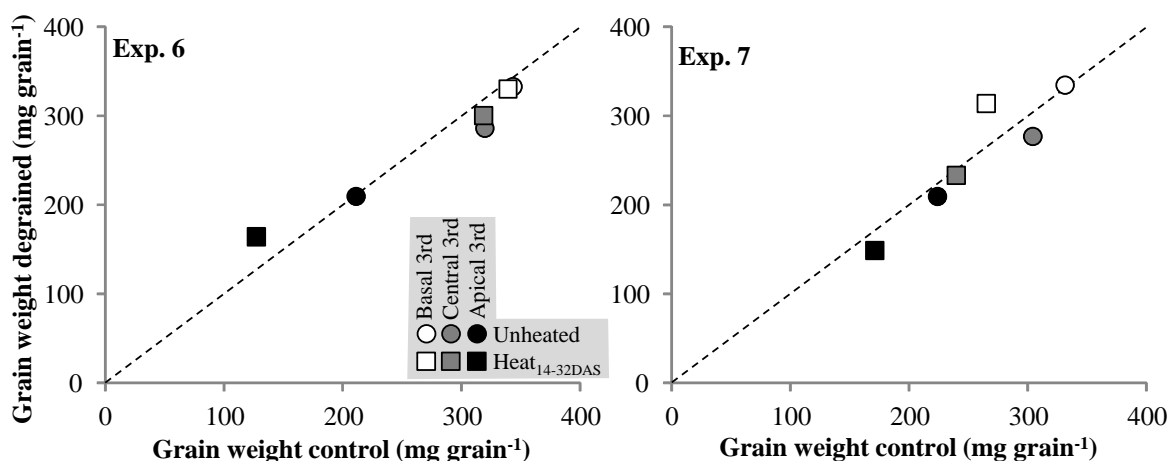
Figure 9. Grain weight of the basal, central and apical thirds of the ears (open, grey and closed symbols, respectively) in the degrained plants plotted against the values corresponding to the control exps. 6 and 7 under heat stress (squares) and unheated conditions (circles). Each data-point is the average of all N fertilisation treatments within each experiment. Dashed lines represent y = x, the 1:1 ratio.

Removing neighbour grains did not increase the weight of the remaining ones in any of the thirds of the ear (Fig. 9), implying that grains from the apical third of the ear are smaller due to constitutive reasons, perhaps the competition during the establishment of the potential grain size made them to have that smaller size potential, and therefore the idea that grain might be theoretically competing for limited photoassimilates during the effective period of grain filling was not supported by the evidences (Figs. 8 and 9).

Again heat stress reduced grain weight at all positions in all experiments and there was no consistent evidence that removing theoretically competing grains would reverse at least in part the penalty in grain weight (Fig. 9); strengthening more the impression that heat directly reduces the capacity of the grains to grow. Degraining did consistently increase grain N concentration under both unheated and heat stressed conditions (Fig. 10).
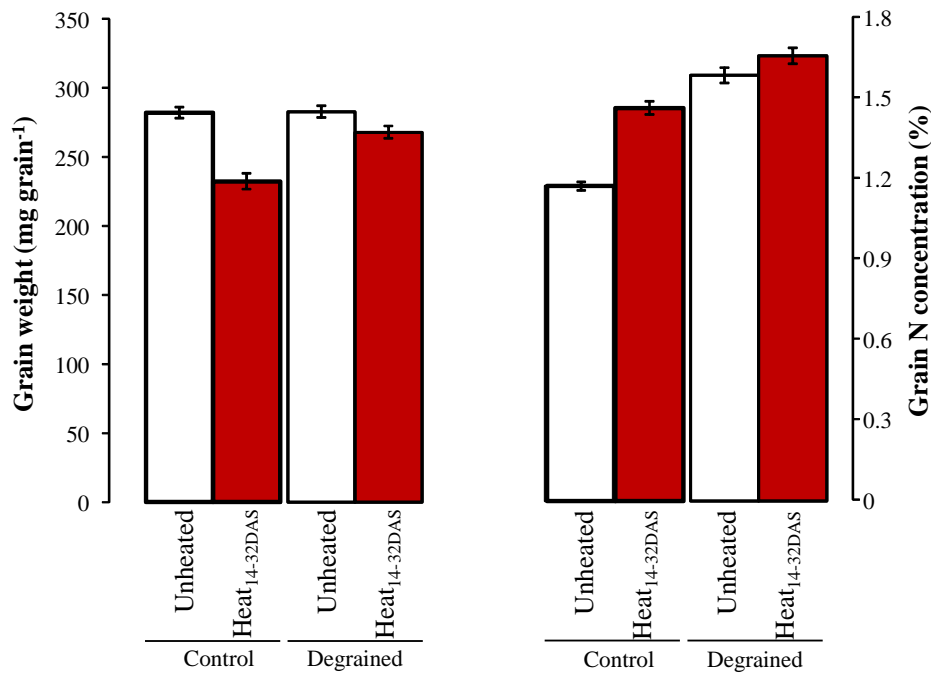
Figure 10. Average weight (left) and grain N concentration (right) of grains from plants which were either unheated or heat stressed during the effective period of grain filling in factorial combination with two different source-sink balances: control, not defoliated, plants and defoliated (65-75%) 15 d after silking. Segments on each bar stand for the standard error of the means.

6.4 Discussion

With the exception of the lack of clear reductions in grain size in response to the defoliation treatment in exp. 7, all our results are in strong agreement with conclusions from the meta-analysis done by Borrás et al., (2004) and with other papers published since that meta-analysis was published. That is grain weight is largely unresponsive to increases in availability of assimilates during the effective period of grain filling (when the potential size of the grains had been established), but strongly diminished by reducing availability of assimilates. Therefore it may be concluded that our results support that grain growth during the effective period of grain filling in maize would be largely limited by the sink-strength (as also concluded by Otegui et al., 1995; Maddonni et al., 1998; Borrás and Westgate, 2006; Gambín et al., 2008), which in turn is determined slightly earlier when the potential size of the grains is defined (Gambín et al., 2006; Borrás and Gambín, 2010), and therefore yield would be strongly related to the sink strength established during the critical period for grain number determination (when grain number and potential grain size are being simultaneously determined). This would be a situation quite similar to that of wheat, in which it has also been repeatedly shown that grain growth is most frequently limited by the sink (Slafer and Savin, 1994; Borrás et al., 2004; Cartelle et al., 2006; Serrago et al., 2013). In fact, it is expected from an evolutionary perspective that grain size would be conservative (Sadras, 2007) and the reproductive output would better adjust to the growing conditions through inexpensively initiating a massive number of primordia which will eventually became grains (Sadras and Slafer, 2012), which is behind the virtually universal positive relationship between yield and grain number (Borrás et al., 2004). An excess of the canopy capacity respect to the growing grains demand during the effective grain filling period is the basic principle to reduce plasticity of grain size, and consequently most grain crops would hardly have yield source-limited during the effective period of grain filling (and in virtually all grain crops yield would be strongly source limited during the critical period for grain number determination; Slafer and Savin, 2006).

The support of the overall conclusion that grain growth is not limited by the source during the effective period of grain filling is based on the unresponsiveness of grain size to increases in the source-sink ratio after the lag phase. In maize, different treatments have been applied to increase the source sink ratio relative to a control (the normal crop), but the

approaches used were always difficult to interpret: the most common approach has spanned from modifying the crop structure (plant density) through controlled pollination to reduce the number of grains set per unit of biomass around silking to thinning plants few weeks after silking. Changes in density and in controlled pollination may also affect grain weight potential and then differences in grain size at maturity may be reflecting more than the degree of source or sink limitation during the effective period of grain filling. Thinning plants after the lag phase is in principle a much cleaner treatment as it affects the potential capacity of plant growth after grain number and the potential size of the grains have been established, but the problem is that depending on the canopy structure of the control a particular thinning treatment may affect more or less the potential increase in source per growing grain. As far as we are aware, the approach designed in the present study offers for the first time in maize, a direct and quantitatively certain increase in source sink balance during the effective period of grain filling. Thus, the corroboration with our data of previous conclusions is not a mere additional evidence but a strong support provided for the first time with a direct approach reaffirming what has been a number of times served from more indirect approaches to increase source per growing grain.

Although the (lack of) response of maize to improved assimilate availability per grain during the effective grain filling period is in line with what has been seen for other cereals, what has always been different between maize and wheat is the sensitivity to the reduced assimilate availability in which wheat is largely unresponsive (Borrás et al., 2004 and references quoted therein) unless the reduction is rather extreme (e.g. shading more than 80% of the incoming radiation during the whole grain filling period: Sandaña et al.; 2009; Serrago et al., 2013), while maize tends to respond with proportional reductions in grain weight even to mild or moderate reductions in potential availability of assimilates (Borrás et al., 2004). This quantitative reduction in grain size with relatively moderate reductions in source-sink ratios could be also potentially interpreted as if in the control situation grains were growing at a co-limitation by source and sink strengths. However, results from the present study would challenge that interpretation, even when we may not be able to offer a more solid alternative.

If the reduction in grain weight in response to defoliation would have been the consequence of grain growth being limited by lack of enough assimilates for all grains to grow close to their potential size it would be expected that the damage would be hierarchical: the dominant grains would be much less affected (or not affected at all) and the weakest grains would be more severely damage. This hierarchical response is not only expected from general biology studies but also empirically determined for maize grain filling since the early work by Daynard and Duncan (1969) studying what determines the maturity of maize grains. In that paper published almost half century ago, Daynard and Duncan (1969) stated that if a stress occurs during the effective grain filling period the grains of the tip of the ear would be those most affected and then a large group will fill only partially and a remaining group will fill as in the non-stressed condition. We are not aware of other studies with defoliations having analysed the response of different populations of grains but in the present study this expected hierarchical damage did not occur: defoliation reduced the final weight of the grains similarly disregarding whether they belonged to the basal, central or apical part of the ears. Definitively abortion of grains is clearly related to source availability (normally grain number is a function of plant growth rate during the critical period; *e.g.* Vega et al., 2001) and definitively the damage is far more obvious in the tip than in the base of the ears, because these florets represent much weaker sinks than the basal ones fertilised earlier as demonstrated since long time ago (Daynard and Duncan, 1969). Then, the reduction in grain size may be related to other reasons than to the competition for limited assimilates. There may be signals associated with the treatments as signals may affect organs similarly, independently of the hierarchies (as temperature does, see below). We are aware of another study in which reduction in source per growing grain was analysed independently for grains in the base and in the tip of the ears, although the treatment was not defoliation but shading 45 % of the incoming radiation imposed from two weeks after silking to maturity (Andrade and Ferreiro, 1996). If the grains would be growing in the control close to a source-limited condition it would be expected a reduction due to increased competition for limited resources in the shaded plants (i) of around 45% (the intensity of shading), or a bit less due to likely contributions from pre-silking reserves (which may not be in maize as relevant as they are in wheat; Borrás et al., 2004); and (ii) more dramatic in grains of the tip than in those of the base of the ears, as the latter would be

stronger competitors for limited resources. Although reductions in grain weight due to shading were always statistically significant (Andrade and Ferreiro, 1996), none of the two premises to conclude that grains in the control plants were growing close to a source limitation were fulfilled. Firstly, the magnitude of the reduction was only marginal (c. 11%) in the first growing season (while in the second growing season the reduction was more relevant though yet less than expected). Secondly and in agreement with our results, the magnitude of the penalty was similar (or even less less) in the weaker grains of the apical part of the ear than in the supposedly dominant grains of the basal part of the ear (see Fig. 2 in Andrade and Ferreiro, 1996). Although we cannot offer a more suitable hypothesis than the one most widely accepted that reduction in grain growth due to (even mild-moderate) reductions in source strength during the effective period of grain filling reflects that in the control the grains would be growing in "a delicate balance between sink and source" (Tollenaar and Daynard, 1982), this might need to be rethought at the light that perhaps something else than purely and simply competition would be behind the reduction in grain size.

Heat stress in this study again seemed to have affected grain size by directly affecting the capacity of the grains to grow. This conclusion was reached in the previous study (Ordoñez et al, Chapter V of this thesis) due to the fact that heat stress reduced grain size even when it increased source-sink ratio (by inducing late abortion of few grains while not affecting much post silking growth). In the present study the direct effect it can be seen because (i) neither defoliation worsened nor degraining diminished consistently the penalty imposed by the heat stress during the effective grain filling period, and (ii) the penalty was similar for grains of different potential size. Thus, the present study further strengthens the conclusion that the effect of heat stress during the period of grain filling is mainly direct (as also concluded by Rattalino Edeira et al., 2014).

6.5 Acknowledgements

6.6 References

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Chapter VII

General discussion

This last chapter of the Thesis is aimed to recap succinctly the main achievements, integrating findings across the experiments/analyses presented in the different experimental chapters in terms of the effect of high temperatures and nitrogen availability (i) on yield and its physiological determinants, (ii) traits for improving nitrogen use efficiency, (iii) yield losses due to high temperature x N, and (iv) crop-physiological bases for temperature x N effects on yield.

7.1 Yield and its determinants

Among hybrids, grain yield was more strongly associated with final grain number per unit area than with the average weigh of the grains, with is very much in line with what has been found by other authors (Otegui and Banhomme, 1998; Andrade et al., 2000, Capristo et al., 2007). In addition, grain yield was realted to biomass at maturity while it was unrelated to biomass at silking, revealing a strong relationship with accumulated biomass from silking to maturity (Fig.8, Chapter II).

The differences between hybrid performances were independent of differences in cycle length but if the comparison is restricted to the average of all short- and long-cycle hybrids, it can be confirmed that the shorter-cycle hybrids had lower production than the long-cycle hybrids. In any case, grain yield for the short-cycle hybrids were reasonably high, making it clear that these short-cycle hybrids may become a feasible alternative for maize production in the Palin of Lleida, when early release of the field is required or when a late sowing is planned. In addition there was a tendency for the performance of the hybrids to be relatively consistent across conditions (location per year). The tendency for higher yield potential genotypes to present better overall performance under lower yielding conditions had been shown in the literature for small grained cereals (Evans and Fischer, 1999; Abeledo et al 2003; Slafer and Araus, 2007) and is expanded here for maize; highlighting the relevance of yield potential even under non-potential conditions.

Within the two groups of hybrids, yield was not consistently related with cycle duration of the hybrids. Despite this general context, the relative response to N fertilization tended to be higher in the long- than in the short-cycle hybrid, implying a higher plasticity may be expected in the long-cycle hybrid. This may be seen positively or otherwise depending on

whether we are considering responsiveness to improved conditions or tolerance to stresses. Thus, analysing responsiveness in terms of the stability analysis it emerges that the PR31N28 tended to be potentially higher yielding whilst Lapopi tended to be more stable (Fig. 5, Chapter III).

Total biomass, LAI and total N uptake at silking was higher for the long-than the short cycle-hybrid at low and warm and similar at high and cold location. These differences found at low altitude disappeared at maturity, when all the values for these variables were similar in both cycle length hybrids. Then end result was that both hybrids achieved similar yields in both locations, implying that for explaining the overall variation in yield growth after silking was more relevant than growth before silking. However it cannot be discarded that part of the differences in growth after silking may respond to differences in sink strength (number of grains and potential size of the grains) that is set around silking (see detailed discussion and references in Chapters V and VI). The interplay between the determination of grain number and potential size of the grains, which might differ strongly between hybrids, may explain that in the present study both yield components explained yield similarly (though the range explored by grain number was a bit wider and therefore may be more responsible than grain size of the yield plasticity explored; see discussion on this issue in Sadras, 2007 and in Sadras and Slafer, 2012).

Expectedly yield was related to the capture of resources than to the efficiency of use of these resources. This is generally the case when variation in resource availability is explored, like in the present study. It is normally the case that when crops are fertilised the growth is affected more than the partitioning and N uptake more favoured than N utilisation efficiency, which is normally reduced in response to improved availability. Although protein percentage is normally negatively related to yield the reason for this negative relationship is that grain growth is largely sink limited while N accumulation in the grains is source limited producing a dilution effect (see Chapter VI for evidences and in depth discussion of the issue). However large changes in availability of resources may simultaneously modify sink strength (through improving grain number and potential size of the grains) as well as source of N (N content in vegetative organs which might be remobilised later), as illustrated in the bi-plot of the principal component analysis offered

(in which GN increased simultaneously with N content in vegetative organs at silking) explaining the positive relationship overall conditions between yield and protein concentration of grains without conflicting with the idea that in concrete conditions N in the grains may be diluted by the amount of C being accumulated during grain filling. This is in fact consistent with that yield was in general related to N uptake and negatively related to N utilisation efficiency, being the later the driving force for the protein concentration in grains (the higher the efficiency in using a certain amount of N for producing yield the more diluted the N accumulated in the grains would be; e.g. Pedro et al., 2011).

Regarding to leaf area production, there were clear differences between locations. Values for this trait were lower in the warm than the cold environment (from *c*. 4.4 to slightly more than 5.1). Also, LAI values were different (P≤0.001) between hybrids, as also found other authors (Borrás et al., 2003; Maddonni and Otegui, 1996). However the differences between long- and short-cycle hybrids could be related to the fact that the long-cycle hybrid produced more leaves than the short-cycle, in agreement with what found since long time ago (Chase and Nanda, 1967). Senescence was clearly delayed by N fertilization. Leaf green area duration in the short-cycle was reduced in *c*.12 %, while in the long-cycle hybrid was *c*. 23 % under no N fertilised conditions. The response of leaf senescence rate (assessed as the loss of green colour) was more markedly in the low than in the high altitude location (Table 4; Fig. 3, Chapter III). This response probably could be explained based in the warmer temperature of low altitude during each growing stage than the high altitude. General senescence during grain filing is related to local growing conditions and the perceived light quantity by the leaf and nitrogen availability (Borrás et al., 2003). This effect was also observed by Tollenaar and Daynard (1978), in a comparison of 10 short-cycle hybrids during two consecutive years.

7.2 Traits for improving nitrogen use efficiency

Identifying traits to phenotype populations for increased NUE would be relevant for future agricultural systems which are expected to yield more grain without increasing (or even with decreasing) use of inputs, such as N fertilizers. As much of the N absorbed by the crop is in the leaves at silking (He et al., 2003; Rajcan and Tollenaar, 1999), the time when yield

is largely determined (Lemcoff and Loomis, 1994); the relationship between yield and SLN at silking may be instrumental for identifying such phenotyping trait. However, for identifying a threshold for phenotyping with a complex trait like this it would be relevant that variation in this threshold were negligible; or at least that no genotypes exhibit a SLNc lower than the threshold for which the breeding program would be selecting for. Otherwise we may erroneously either (i) discard genotypes with SLN values lower than the threshold assuming they would not maximize yield in these conditions (for genotypes with a SLNc lower than the threshold proposed), or (ii) select genotypes with SLN values equal to the threshold assuming wrongly that they would maximize yield in these conditions (for genotypes with a SLNc higher than the threshold proposed). For that reason, determining whether there is noticeable genetic variation in SLNc is relevant to conclude on whether a threshold SLN can be used to phenotype to improve yield through increasing NUE.

Substantial variation in SLNc (roughly from 1 to 2 gN m^{-2}) was found comparing 11 commercial hybrids. We did this comparison being conscious that the population analyzed would be very conservative but would represent fairly the type of material breeders work with when trying to improve yield, NUE or any other complex trait. It would be expected that a more variable population may express even a larger degree of variation in SLNc. Therefore, although it remains true that for improving NUE it would be very positive to select for genotypes with SLN = SLNc when grown under non-optimal N availability levels, there seems to be considerable intraspecific variation in SLNc and therefore it would be hardly possible to use a particular threshold for SLN to phenotype plants which would be maximizing yield in those conditions.

7.3 Yield losses due to high temperature x N

In this Thesis it was found in agreement with a large body of literature, that yield of maize (as well as that of other cereals) is highly responsive to N fertilisation (positively) and to heat stress (negatively). However, no studies seem to have been conducted to quantify the likely interaction between these two factors. We found, and as far as we are aware for the first time in maize grown in field conditions, that the losses in yield in response to high temperature were magnified by the availability of N. This may require, more and more

often, that the doses of N fertilisation be fine-tuned to avoid yield penalties derived from N stress but at the same time to avoid higher yield penalties in the event of heat stress. The results from this Thesis are focused on yield, but the relevance of considering the interaction uncovered would be even more noticeable if we consider the gross margin, considering the costs of the fertilisation.

As mentioned above, these are the first results from field grown maize on the interactions between N fertilisation and heat on maize yield. There are only very limited data available from other cereals. These few results are in agreement with the conclusion we reached in the present study: the yield penalty produced by heat stress was higher under N fertilised treatments than in the unfertilised. This was reported for wheat (Altenbach et al., 2003; Zahedi et al. 2004; Dupont et al., 2006) as well as for barley (Passarella et al., 2008). The trade-off in grain protein produced by heat stress in the present study is also in line with that reported for wheat by Altenbach et al. (2003) and for barley by Passarella et al. (2008). Therefore, the strength of the conclusions of the present study are no limited to the number of field experiments in which they are based but also in that the limited amount of evidences of this sort of interaction available in the literature for other cereals are all in line with them.

Two further elements of strength in our study are the experimental approach and the magnitude of the treatments. Regarding the approach, all our results come from field experiments, when the vast majority of knowledge on the effects of high temperature is based from extrapolations from more or less controlled conditions. As extrapolation of conclusions from controlled conditions may be difficult to accept straightforwardly (Passioura, 2006), counting with results from field experiments is essential before conclusions could be extrapolated. We are only aware of very few other cases in which high temperature treatments were imposed to maize crops in the field (in all cases from the lab of Prof. Otegui; e.g. Rattalino Edreira et al., 2011 and Cicchino et al., 2010a,b), with an approach similar to that we used in the present study, and that has been used in the past for small-grained cereals (which are far easier to manage due to the size of the plots; e.g. Borghi et al., 1995; Rawson, 1995; Savin et al., 1996; Calderini et al., 1999; Passarella et al., 2002; Ugarte et al., 2007). Regarding the magnitude of treatments, the extrapolation of

results can many times be difficult when treatments are too extreme (which is always attractive to researchers as it facilitates detection of effects). N fertilisation treatments were well within normal rates used by farmers in the region (e.g. Berenguer et al., 2009) and temperature increases were not only reasonable in magnitude but also in the way they were imposed: a heating system based on the greenhouse effect allowing a steady increase during de morning and early afternoon followed by a gradual decrease every afternoon-evening to maximum values averaging relatively likely temperatures in realistic hot days of most temperate regions worldwide (daily maximum temperatures, averaging across the canopy, normally below 40 ℃). This is critically important as the effect of heat not only depends on the magnitude of the high temperature used but also on the rate of change in temperatures for imposing the stress (Wahid et al., 2007). The relevance of the rate of increase from minimum to maximum temperatures to have trustworthy conclusions was already evidenced both in small grained cereals (Savin et al., 1997) and in maize (Crafts-Brandner and Salvucci, 2002). Suddenly imposed treatments, unlike what happens in days with stressful maximum temperatures, do not allow for acclimation processes to take place and therefore might overestimate the magnitude of the penalties actually expected in reality.

Even when the imposed heat stress was relatively mild (maximum temperatures not beyond what can be expected in reality on hot days of temperate areas around silking and grain filling of maize, in most cases only for a few days, and within each day with slow rates of increase) the yield penalty was dramatic when it coincided in full or in part with the critical period for yield determination (c. 30 d bracketing silking).

Although the penalty was less severe than when the stress was imposed during the critical period of grain number determination, heat stress during the effective grain filling period did also affect yield. The effect was through affecting not only the average weight of the growing grains but also the number of grains, although we attempted to impose this treatment after the number of grains has been supposedly fixed (15 d after silking).

The effect of N on emphasising the penalties seemed not to be a direct effect of this nutrient but and indirect effect through affecting growth. The fact that the results of other independent studies not having N fertilisation as a treatment fit well the same relationship with data of the present study (Fig. 12, left panel) provides further support to the conclusion

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that the effect of N on affecting the magnitude of the loss was indirect through plant growth, which is in agreement with previous results both in maize (Andrade et al., 2002), and in other cereals (Fischer, 1993; Demotes-Mainard and Jeuffroy, 2004; Prystupa et al., 2004; Ferrante et al., 2010), although exceptions for some genotypes may be found (e.g. D'Andrea et al., 2008).

7.4 Crop-physiological bases for temperature x N effects on yield

The extreme yield sensitivity to heat stress during the critical period was not related to changes in phenology caused by high temperatures, as in fact temperature treatments hardly affected time to silking in the present study. It is likely that this lack of effect is revealing that the temperature on the heat-stressed plots would have been above the optimum temperature for at least a significant part of the day. Estimates of relatively low optimum temperatures for the critical period ranging from 30 to 36 ºC were recently reported by Cicchino et al. (2010a) and are in line with optimum temperatures estimated for leaf appearance rates in maize (Kim et al., 2007). Optimum temperatures within the range that plants can be exposed to in realistic field conditions had also been reported for wheat (Slafer and Rawson, 1995). Heat stress does affect the availability and viability of pollen (Schoper et al., 1986; 1987; Mitchell and Petolino, 1988) as pollen desiccation is a function of air temperature and pollen viability decreased linearly with pollen humidity (Aylor, 2003; Fonseca and Westgate, 2005). However, in the present experiments none of the highly expected effects of heat on pollen availability and viability were part of the causes of reduction in grain number when plants were heat-stressed during the critical period as the silks in each of the pants were pollinated with fresh pollen. The fact that yield penalties produced by heat stress were not mitigated by pollinating heated plants with fresh pollen had been already reported (Cicchino et al., 2010b); indicating that the major avenue by which heat affected yield dramatically was indirectly through affecting growth of the ears and/or directly through affecting grain abortion. N fertilisation did not consistently affect phenology either. D'Andrea et al. (2009) compared developmental attributes of inbred lines and hybrids to extremely contrasting N fertilisation regimes (0 and 400 Kg_N ha^{-1}) finding no differences in final leaf number and only relatively marginal advances in silking

(averaging 20 and 40 ºC d for lines and hybrids, respectively), with significant genotype x N interactions. This is more or less in line with a lack of consistent evidences from field experiments in other cereals (Hall et al., 2014). Indeed, it seemed that the collapse in yield produced by the heat stress during the critical period was of such magnitude because of concomitantly occurring direct and indirect effects on grain number loss, and N fertilisation increased the magnitude of both types of effects.

The indirect effects operated through reductions in plant growth during the critical period, which might be reflecting reductions in radiation use efficiency (Cicchino et al., 2010b; Rattalino Edeira and Otegui, 2012); as radiation interception would hardly be affected by relatively short periods of high maximum temperatures starting well after maximum radiation interception has been reached. Important reductions in radiation use efficiency would be expected as high temperatures would concurrently reduce photosynthesis and increase respiration (Penning de Vries et al., 1979; Tollenaar et al., 1989; Crafts-Brandner and Salvucci, 2002; Sinsawat et al., 2004; Hay and Porter, 2006; Kim et al., 2007). This effect on plant growth was complemented with a reduction in partitioning of that growth to the growing juvenile ear.

The direct effects operated through reductions in fruiting efficiency beyond those predictable due to reductions in ear partitioning: heat stressed plants set a much smaller number of grains per unit plant growth during the critical period than the plants which were unheated. No clear mechanisms can be proposed at this stage for these direct effects inducing abortion of pollinated female florets, but the fact that abiotic stresses may induce fertile florets to be abortive, even if grain set is warranted by manipulating pollination, has been already reported (Basetti and Westgate, 1993; Otegui et al., 1995; Cárcova and Otegui, 2001) and possible mechanisms suggested (Barnabás et al., 2008). In the study by Rattalino Edeira et al. (2012), the main damage produced by heat stress during the critical period was also associated with direct more than with indirect effects. These direct effects are largely responsible for the important reduction in harvest index caused by heat stress during the critical period (as the collapse in yield was related to a relatively modest reduction in total growth and N uptake). This effect of heat stress on harvest index is rather

common (Ferris et al., 1998; Craufurd et al., 2002; Cicchino et al., 2010b; Rattalino Edeira and Otegui, 2012).

Due to the large direct effects of heat stress during the critical period on grain number through reducing considerably fruiting efficiency, it was expected a likely increase in average grain size due to two physiological mechanisms. However, we did not find any consistent increase in grain weight compensating, at least partially, the large reduction in grain number produced by the heat during the critical period. A recent paper also showed evidences supporting that heat stress during the critical period of grain number determination, even when reducing considerably fruiting efficiency, does only marginally affect grain size (Rattalino Edeira et al., 2014). It might be possible then to speculate that there would be a direct effect of temperature on potential size of the grains which is not detectable in the present (and the other above-mentioned) study because it would have been counterweighed by of the potential increase produced by the large improvement in plant growth per grain set.

The lack of any significant feed-forward effect of heat during the critical period on grain growth was also evidences by the fact that, in relative terms, the reductions in yield were quantitatively mirrored by increases in grain protein concentration. Thus the grains set in the plants subjected to heat stress during the grain determination period were not impaired in their capacity to accumulate N, which is normally strongly limited by the source. That is why the effects of the combinations of high temperature x N fertilisation treatments on N utilisation efficiency were very strongly negatively related to grain protein concentration. Similar results were shown for wheat (Pedro et al., 2011; Ferrante et al., 2012) in the absence of heat stress.

When the heat stress was imposed 15 d after silking there was still a significant reduction in grain number, though much more modest than when the heat was imposed in the critical period for grain number determination. This means that it is likely that, at least for the hybrids and background conditions used in the present study and in that carried out by Rattalino Edreira et al. (2012), which were quite different, the critical period for grain number determination seemed to have actually finished at least a bit later than 15 d after silking.

The analysis of the effect of heat stress treatments during grain filling on biomass accumulation during silking-maturity suggest that the effect on grain weight was not mediated by a limitation of the source strength. The analysis of the effects on the post-silking growth per grain set reinforces the idea that the effects were direct on the capacity of the grains to grow: due to the reduction imposed in grain number, biomass accumulation per grain during post-silking was higher in the heat-stressed than in the unheated plants. Furthermore, the heat stress was mild enough not to allow a clear and consistent acceleration of chlorophyll loss from the leaves. The conclusion that the effect was mainly direct on the capacity of the grains to grow is commensurate with the idea that the effective grain filling is largely sink-limited (Borrás et al., 2004; Gambín et al., 2006; 2008).

The direct effect of temperature may be related to a diminishing enzymatic activity responsible for starch synthesis in the grains (Jenner, 1994). For instance, at high temperature grains could hardly grow even when the concentration of soluble sugars was high (Jones et al., 1981). Thus high temperature effect on grain size cannot be reversed, nor diminished, by increasing the source-sink ratio (Slafer and Miralles, 1992). Another direct effect of heat stress on grain weight might be through increasing the rate of water loss from the grain during the first half of the effective grain filling period (Rattalino Edeira et al., 2014). The dynamics of water content is quite relevant in establishing the final weight of the grains (Borrás et al., 2003; Gambín et al., 2007; Borrás and Gambín, 2010) and treatments affecting this dynamics might impair normal grain growth beyond any effects on availability of soluble sugars to synthesise starch.

With the exception of the lack of clear reductions in grain size in response to the defoliation treatment in exp. 7, all our results are in strong agreement with conclusions from the meta-analysis done by Borrás et al., (2004) and with other papers published since that meta-analysis was published. That is grain weight is largely unresponsive to increases in availability of assimilates during the effective period of grain filling (when the potential size of the grains had been established), but strongly diminished by reducing availability of assimilates. Therefore it may be concluded that our results support that grain growth during the effective period of grain filling in maize would be largely limited by the sink-strength (as also concluded by Otegui et al., 1995; Maddonni et al., 1998; Borrás and Westgate,

2006; Gambín et al., 2008), which in turn is determined slightly earlier when the potential size of the grains is defined (Gambín et al., 2006; Borrás and Gambín, 2010), and therefore yield would be strongly related to the sink strength established during the critical period for grain number determination (when grain number and potential grain size are being simultaneously determined).

The support of the overall conclusion that grain growth is not limited by the source during the effective period of grain filling is based on the unresponsiveness of grain size to increases in the source-sink ratio after the lag phase. In maize, different treatments have been applied to increase the source sink ratio relative to a control (the normal crop), but the approaches used were always difficult to interpret: the most common approach has spanned from modifying the crop structure (plant density) through controlled pollination to reduce the number of grains set per unit of biomass around silking to thinning plants few weeks after silking.. As far as we are aware, the approach designed in the present study offers for the first time in maize, a direct and quantitatively certain increase in source sink balance during the effective period of grain filling. Thus, the corroboration with our data of previous conclusions is not a mere additional evidence but a strong support provided for the first time with a direct approach reaffirming what has been a number of times served from more indirect approaches to increase source per growing grain.

Although the (lack of) response of maize to improved assimilate availability per grain during the effective grain filling period is in line with what has been seen for other cereals, what has always been different between maize and wheat is the sensitivity to the reduced assimilate availability in which wheat is largely unresponsive (Borrás et al., 2004 and references quoted therein) unless the reduction is rather extreme (e.g. shading more than 80% of the incoming radiation during the whole grain filling period: Sandaña et al.; 2009; Serrago et al., 2013), while maize tends to respond with proportional reductions in grain weight even to mild or moderate reductions in potential availability of assimilates (Borrás et al., 2004). This quantitative reduction in grain size with relatively moderate reductions in source-sink ratios could be also potentially interpreted as if in the control situation grains were growing at a co-limitation by source and sink strengths. However, results from the

present study would challenge that interpretation, even when we may not be able to offer a more solid alternative.

If the reduction in grain weight in response to defoliation would have been the consequence of grain growth being limited by lack of enough assimilates for all grains to grow close to their potential size it would be expected that the damage would be hierarchical: the dominant grains would be much less affected (or not affected at all) and the weakest grains would be more severely damage. This hierarchical response is not only expected from general biology studies but also empirically determined for maize grain filling since the early work by Daynard and Duncan (1969) studying what determines the maturity of maize grains. In that paper published almost half century ago, Daynard and Duncan (1969) stated that if a stress occurs during the effective grain filling period the grains of the tip of the ear would be those most affected and then a large group will fill only partially and a remaining group will fill as in the non-stressed condition. We are not aware of other studies with defoliations having analysed the response of different populations of grains but in the present study this expected hierarchical damage did not occur: defoliation reduced the final weight of the grains similarly disregarding whether they belonged to the basal, central or apical part of the ears. Definitively abortion of grains is clearly related to source availability (normally grain number is a function of plant growth rate during the critical period; *e.g.* Vega et al., 2001) and definitively the damage is far more obvious in the tip than in the base of the ears, because these florets represent much weaker sinks than the basal ones fertilised earlier as demonstrated since long time ago (Daynard and Duncan, 1969). Then, the reduction in grain size may be related to other reasons than to the competition for limited assimilates. There may be signals associated with the treatments as signals may affect organs similarly, independently of the hierarchies (as temperature does, see below). We are aware of another study in which reduction in source per growing grain was analysed independently for grains in the base and in the tip of the ears, although the treatment was not defoliation but shading 45 % of the incoming radiation imposed from two weeks after silking to maturity (Andrade and Ferreiro, 1996). If the grains would be growing in the control close to a source-limited condition it would be expected a reduction due to increased competition for limited resources in the shaded plants (i) of around 45% (the intensity of shading), or a bit less due to likely contributions from pre-silking reserves

(which may not be in maize as relevant as they are in wheat; Borrás et al., 2004); and (ii) more dramatic in grains of the tip than in those of the base of the ears, as the latter would be stronger competitors for limited resources. Although reductions in grain weight due to shading were always statistically significant (Andrade and Ferreiro, 1996), none of the two premises to conclude that grains in the control plants were growing close to a source limitation were fulfilled. Firstly, the magnitude of the reduction was only marginal (c. 11%) in the first growing season (while in the second growing season the reduction was more relevant though yet less than expected). Secondly and in agreement with our results, the magnitude of the penalty was similar (or even less less) in the weaker grains of the apical part of the ear than in the supposedly dominant grains of the basal part of the ear (see Fig. 2 in Andrade and Ferreiro, 1996). Although we cannot offer a more suitable hypothesis than the one most widely accepted that reduction in grain growth due to (even mild-moderate) reductions in source strength during the effective period of grain filling reflects that in the control the grains would be growing in "a delicate balance between sink and source" (Tollenaar and Daynard, 1982), this might need to be rethought at the light that perhaps something else than purely and simply competition would be behind the reduction in grain size.

7.5 References

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Chapter VIII

General conclusions

The general objective of this thesis was to assess the genotypic variability in yield components and the susceptibility of yield determinants to thermal stress and nitrogen availability in maize. A strong point of the present thesis was that the issue was explored throughout 7 field experiments, 12 maize hybrids of different maturity type, different nitrogen availabilities sown at farmer's fields during 4 consecutive experimental years. Moreover, the high temperature stresses were imposed at the field: the designated area for the treatments was enclosed with transparent polyethylene film (100 μm thickness) mounted in wood structures of 3-3.5 m height.

The main results achieved were:

(i)   The differences between hybrid performances were independent of differences in cycle length but if the comparison is restricted to the average of all short- and long-cycle hybrids, it can be confirmed that the shorter-cycle hybrids had lower production than the long-cycle hybrids. Even though, grain yield for the short-cycle hybrids were reasonably high. A valuable aspect is that there was a tendency for higher yielding hybrids in a condition (location per year) tended to be the highest performance in another location or in another year (Chapter II).

(ii)   Among all hybrids, grain yield was positievely associated with final grain number per unit area more than with grain weight as found by other authors. In addition, grain yield was related to total biomass at silking revealing a strong relationship with accumulated biomass from silking to maturity and to nitrogen uptake at maturity (Chapter II and III).

(iii)   As future genetic gains in yield would be expected to include improvements in N use efficiency (NUE), SLNcritical at siliking was identify as a surrogate to identified differences among hybrids. The results in Chapter IV, indicated that it does not seem appropriate to phenotype for a particular value of SLNcritical as any particular threshold might bring about selected lines with either a yield potential lower than possible and/or with a relatively lower than possible NUE.

(iv)   Overall the range of conditions yield was more strongly affected by capture, than by partitioning or efficiency of use, of resources and the in this context grain yield was positively related to both of its components similarly (even though grain number was more determinant than grain weight for the plasticity of yield) as well as to grain protein concentration; but these relationships are actually driven by the huge environmental range (years x locations x N regimes) explored. In further chapters the physiology of determination of yield and the relationships with these other traits will be further explored (Chapter III).

(v)   In particular, the negative relationship between yield and NUE in the context of the wide range of conditions does not preclude the awareness that future hybrids shall be more NU Efficient and that ways to select for improved NUE must be developed for future agricultural systems in which N is expected to be less freely available while yields must keep increased (Chapter III).

(vi)   It was proved for the first time in maize that the sensitivity of yield to heat stress was increased by N fertilisation. This conclusion is based on field experiments with treatments of a magnitude well within expected variation in realistic conditions. The effect was through affecting the capacity of the plants to set grains and to a lesser extent to allow grain weight to be maximised; and it was independent of any (potentially additional) effects on either uncoupling anthesis and silking or on pollen amount and viability. The influence of N fertilisation on yield was quite universal: it was evident for reductions in grain number (by far the most critical component responsible for the penalties imposed by heat stress) and in average grain weight; and it was clear through both indirect and direct mechanisms of penalising yield components (Chapter V).

(vii) To determine the degree of source-sink-limitations for grain growth, a novel approach to determine to what degree grains compete for limited resources during the effective period of grain filling, once their potential size has been established. Heat stress seemed to have affected grain size by directly affecting the capacity of the grains to grow due to the fact that heat stress reduced grain

size even when it increased source-sink ratio (by inducing late abortion of few grains while not affecting much post silking growth). The direct effect it can be seen because neither defoliation worsened nor degraining diminished consistently the penalty imposed by the heat stress during the effective grain filling period, and the penalty was similar for grains of different potential size. Thus, the present study further strengthens the conclusion that the effect of heat stress during the period of grain filling is mainly direct.