

CHAPTER 1

Legumes and breeding under abiotic stress: An overview

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1.1 Introduction

The present world population of 7.2 billion is expected to reach 9.6 billion by the middle of the 21st century due to the high growth rate, particularly in developing countries. There is a need to produce about 70% more food to feed this excessive population (Varshney & Roorkiwal, 2013).

Legumes belong to the family Fabaceae/Leguminosae (with about 700 genera and 18,000 species). Legume crops can be divided into two groups according to their ability to grow in different seasons, namely cool season food legumes and warm or tropical season food legumes (Miller *et al.*, 2002; Toker & Yadav, 2010). The cool season food legumes include broad bean (*Vicia faba*), lentil (*Lens culinaris*), lupins (*Lupinus* spp.), dry pea (*Pisum sativum*), chickpea (*Cicer arietinum*), grass pea (*Lathyrus sativus*) and common vetch (*Vicia sativa*) crops (FAOSTAT 2009; Andrews & Hodge, 2010). These are among the world's oldest cultivated plants (Materne *et al.*, 2011). Dry pea, chickpea, broad bean and lentil are the four major cool season grain legume crops produced for human consumption. They are grown on all continents except Antarctica. Lupin species – e.g. *Lupinus albus* (white lupin) and *Lupinus luteus* (yellow lupin) – and vetches – in particular, common vetch – are important for animal feed (Andrews & Hodge, 2010). On the other hand, the warm season food legumes include pigeon pea (*Cajanus cajan*), cowpea (*Vigna unguiculata*), soybean (*Glycine max* L.), mung bean (*Vigna radiata* var. *radiata*) and urd bean (*Vigna mungo*) crops, which are mainly grown in hot and humid climatic

conditions. Warm season food legumes are popular in different parts of world; for example, pigeon pea is mainly grown in India and African countries, cowpea and soybean are important crops in the USA, while mung bean and urd bean are important crops in Southeast Asian countries, especially in the Indian sub-continent (Singh *et al.*, 2011).

Legumes rank third after cereals and oilseeds in world production and have major effects on the environment, agriculture, and animal and human nutrition and health (Graham & Vance, 2003; Dita *et al.*, 2006; Mantri *et al.*, 2013). Legumes are a primary source of amino acids and provide around one-third (20–40%) of all dietary protein (Zhu *et al.*, 2005; Kudapa *et al.*, 2013). Legumes produce secondary metabolic compounds that can protect the plant against pathogens and pests (Kudapa *et al.*, 2013).

Legumes are second to cereals in providing food for humans worldwide (Kamal *et al.*, 2003; Ashraf *et al.*, 2010; Kudapa *et al.*, 2013). In comparison with cereal grains, legume seeds are rich in protein, and thus are a source of nutritionally rich food (Ahlawat *et al.*, 2007; Ashraf *et al.*, 2010; Kudapa *et al.*, 2013). Grain legumes such as chickpea, pigeon pea, cowpea, dry pea, lentil, mung bean, urd bean, bean (*Phaseolus vulgaris* L.), broad bean and grass pea are the main source of dietary protein for vegetarians, and are an integral part of the daily diet in several forms worldwide. In addition, grain legumes, predominantly peanut (*Arachis hypogaea* L.) and soybean are also major sources for vegetable oil, providing more than 35% of the world's processed vegetable oil (Sharma *et al.*, 2010).

Legumes play an important role in diet and they are often referred to as 'poor man's meat'. Legumes are an important source of protein, oil, fibre and micronutrients, and play a vital role in cropping cycles due to their ability to fix atmospheric nitrogen (El-Enany *et al.*, 2013; Mantri *et al.*, 2013).

Under conducive environmental conditions, legumes develop symbiotic associations with arbuscular mycorrhizal (AM) fungi, leading to the formation of sites of phosphorus nutrient exchange called arbuscules (Parniske, 2008; Mantri *et al.*, 2013).

Biological fixation of nitrogen (N) is considered more ecofriendly than industrial N fixation because the NH_3 produced in the former process is readily assimilated into organic forms by the plant (Valentine *et al.*, 2011). Biological nitrogen fixation (BNF) in legume nodules occurs with differentiated forms of rhizobia, termed bacteroids, within specialized structures called symbiosomes, inside the host plant cells (Arrese-Igor *et al.*, 2011). Thus, these symbiotic associations have strongly driven the investigation and application of biotechnology tools for legumes (Dita *et al.*, 2006).

It is estimated that crops grown on 90% of arable lands experience one or more environmental stresses. Abiotic stress causes more than 50% of crop loss worldwide (Rasool *et al.*, 2013; Rodziewicz *et al.*, 2014). 'Abiotic stress' is a broad term that includes multiple stresses (drought, waterlogging, salinity, heat, chilling and mineral toxicities) and negatively affects the adaptability and yield of legumes. Application of biotechnology tools to legume crops can help in solving or reducing the problems resulting from abiotic stress.

This chapter aims to review the main abiotic stresses that have a negative impact on the production of some important food legumes. It also summarizes the selection criteria and available genetic resources for stress resistance under abiotic stress conditions.

1.2 Legumes under abiotic stress

1.2.1 Legumes under drought

Drought is a type of water stress that is imposed due to lack of rainfall and/or inadequate irrigation. About 60% of all crop production suffers from drought conditions (Grant, 2012; Naeem *et al.*, 2013). For legumes, drought stress has adverse effects on total biomass, pod number, seed number, seed weight and quality, and seed yield per

plant (Toker *et al.*, 2007b; Charlson *et al.*, 2009; Khan *et al.*, 2010; Toker & Mutlu, 2011; Impa *et al.*, 2012; Hasanuzzaman *et al.*, 2013; Pagano, 2014). Drought alone resulted in about a 40% reduction in soybean yield (Valentine *et al.*, 2011). Faba bean and pea are known to be drought-sensitive, whereas lentil and chickpea are known as drought-resistant genera (Toker & Yadav, 2010). Singh *et al.* (1999) arranged warm season food legumes in increasing order of drought tolerance: soybean < blackgram < greengram < groundnut < Bambara nut < lablab < cowpea. Sinclair and Serraj (1995) reported that legumes such as faba (broad) bean, pea and chickpea export amides (principally asparagine and glutamine) in the nodule xylem are generally more tolerant to drought stress than cowpea, soybean and pigeon pea, which export ureides (allantoin and allantoic acid).

The symbiotic nitrogen fixation (SNF) rate in legume plants rapidly decreased under drought stress due to (i) the accumulation of ureides in both nodules and shoots (Vadez *et al.*, 2000; Charlson *et al.*, 2009), (ii) decline in shoot N demand, (iii) lower xylem translocation rate due to a decreased transpiration rate, and (iv) decline of metabolic enzyme activity (Valentine *et al.*, 2011). Several reports have indicated that drought stress led to inhibition in nodule initiation, nodule growth and development as well as nodule functions (Vadez *et al.*, 2000; Streeter, 2003; Valentine *et al.*, 2011). The decrease in SNF under drought conditions was associated with the reduction of photosynthesis rate in legumes (Ladrera *et al.*, 2007; Valentine *et al.*, 2011).

In many nodules of legumes, water stress resulted in stimulation of sucrose and total sugars (González *et al.*, 1995, 1998; Ramos *et al.*, 1999; Streeter, 2003; Gálvez *et al.*, 2005; Valentine *et al.*, 2011). This was consistent with a study on pea mutants, which showed that sucrose synthase (SS) is essential for normal nodule development and function (Craig *et al.*, 1999; Gordon *et al.*, 1999).

Drought stress induces oxidative damage in legumes and this has a harmful effect on nodule performance and BNF (Arrese-Igor *et al.*, 2011). Some reports suggest that nodules having an increment in enzymatic antioxidant defence can display a higher tolerance to drought/salt stress in common bean (Sassi *et al.*, 2008) and chickpea (Kaur *et al.*, 2009). In addition to this, Verdoy *et al.* (2006) reported improved resistance to drought stress in *Medicago truncatula* by overexpression of Δ -pyrroline-5-carboxylate synthetase resulting in accumulation of high proline levels.

Generally, the mechanisms of drought tolerance include (i) escape, (ii) avoidance, or (iii) resistance (Ishitani *et al.*, 2011; Toker & Mutlu, 2011; Impa *et al.* 2012; Rapparini & Peñuelas, 2014). There are several screening and selection techniques for drought tolerance in food legumes; however, few techniques have been successful under field conditions (Toker & Mutlu, 2011):

- 1 Line source sprinkler irrigation systems (Saxena *et al.*, 1993).
 - 2 Root trait characteristics (root length, root density, root biomass, root length density; Serraj *et al.*, 2004) and the 'root-box pin board' method (Singh & Matsui, 2002).
 - 3 Delayed sowing strategy (Singh *et al.*, 1997).
 - 4 Comparison of lines under non-stressed and stress conditions by defined formula (Silim & Saxena, 1993; Toker & Cagiran 1998).
 - 5 Rain-out shelter tunnels (Abdelmula *et al.*, 1999; Amede *et al.*, 1999; Link *et al.*, 1999).
- [Methods 1–5 can be useful for large-scale screening, but are labour and time consuming (Toker & Mutlu, 2011).]
- 6 Delayed canopy wilting (DCW) in soybean (Charlson *et al.*, 2009).
 - 7 Delayed leaf senescence (DLS) trait in cowpea (Hall *et al.*, 2002).
 - 8 Leaf pubescence density (LPD) in soybean for drought-prone environments (Du *et al.*, 2009).
 - 9 Recovery ability after wilting (RAW) in chickpea (Toker *et al.*, 2007b).
 - 10 The use of carbon isotope discrimination ($\Delta^{13}\text{C}$) in screening. This is described for some food legumes (Stoddard *et al.*, 2006; Khan *et al.* 2010), but it incurs high costs per sample (Toker & Mutlu, 2011).

1.2.2 Legumes under salinity

Salinity is a major abiotic stress limiting germination, plant vigour and yield of agricultural crops especially in arid and semi-arid regions (Munns & Tester, 2008; Abdel Latef & Chaoxing, 2011; Aggarwal *et al.*, 2012; Ahmad & Prasad 2012a, 2012b; Porcel *et al.*, 2012; Kapoor *et al.*, 2013; Abdel Latef & Chaoxing, 2014). Approximately 20% of irrigated land worldwide currently is affected by salinity, particularly in arid and desert lands, which comprise 25% of the total land area of our planet (Yeo, 1999; Rasool *et al.*, 2013). High salinity affects plants in several ways: water stress, ion toxicity, nutritional disorders,

oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, and genotoxicity (Hasegawa *et al.*, 2000, Munns, 2002; Zhu, 2007; Shanker & Venkateswarlu, 2011; Gürsoy *et al.*, 2012; Djanaguiraman & Prasad, 2013). Together, these effects reduce plant growth, development and survival (Rasool *et al.*, 2013; Hameed *et al.*, 2014).

Food legumes are relatively salt sensitive compared with cereal crops, thus farmers do not consider growing food legumes in salinized soils (Saxena *et al.*, 1993; Toker & Mutlu, 2011; Egamberdieva & Lugtenberg, 2014). The sensitivity in legumes may be due to salt affecting bacterial activity and nitrogen fixation (Materne *et al.*, 2007; Toker *et al.*, 2007a; Toker & Mutlu, 2011; Egamberdieva & Lugtenberg, 2014). Salt stress led to reduction in shoot growth of soybean, chickpea, pea, faba bean and mung bean plants (Elsheikh & Wood, 1990, 1995; Delgado *et al.*, 1994; Hussain *et al.*, 2011; Saha *et al.*, 2010; Rasool *et al.*, 2013).

The response of BNF in contrasting tolerance lines of *Medicago ciliaris* to salt stress did not show a clear trend in relation to nodule carbohydrate metabolism (Ben-Sala *et al.*, 2009). Nodules of common bean (Sassi *et al.*, 2008) and chickpea (Kaur *et al.*, 2009) display a higher tolerance to osmotic/salt stress due to increased enzymatic antioxidant defence (Arrese-Igor *et al.*, 2011).

Salinity stress significantly decreased the activities of nitrogenase and phosphate enzymes (acid and alkaline) in faba bean (Rabie *et al.*, 2005; Hussain *et al.*, 2011). The effect of salinity stress on growth and some metabolic activities of mung bean was investigated by Saha *et al.* (2010). They concluded that salinity stress suppressed the early growth of mung bean seedlings. Salinity also damaged the photosynthetic machinery by causing reduced chlorophyll content, and also induced the accumulation of proline, malondialdehyde (MDA) and H_2O_2 in roots and leaves of mung bean plants. Furthermore, salinity stress caused increments in the activity of superoxide dismutase (SOD), catechol peroxidase (CPX) and catalase (CAT) in root and leaves of mung bean plants. Recently, Rasool *et al.* (2013) reported that tolerance of chickpea genotypes (SKUA-06 and SKUA-07) to salinity seems to be related to the efficiency of the enzymatic antioxidants SOD, CAT, ascorbate peroxidase (APX) and glutathione reductase (GR) against accumulation of reactive oxygen species (ROS), which would maintain the redox homeostasis and integrity of cellular components.

Several criteria have been used in screening for salinity tolerance including germination, radicle length, dry weight production, shoot length, cell survival, plant biomass, nodulation, number of pods, grain yield and K^+/Na^+ ratio (Toker *et al.*, 2007a; Flowers *et al.*, 2009; Toker & Mutlu, 2011).

1.2.3 Legumes under waterlogging

Waterlogging occurs when water enters the soil faster than it can drain away under gravity. Waterlogging is a major abiotic factor causing losses in food legumes (Toker & Mutlu, 2011; Ashraf, 2012; El-Enany *et al.*, 2013). It negatively affected germination, seedling emergence, root and shoot growth, and plant density by up to 80%, besides causing seedling diseases (Toker & Mutlu, 2011).

When mung bean plants were subjected to waterlogging stress, the activities of various enzymatic antioxidants such as SOD, CAT, APX and GR decreased markedly (Ahmed *et al.*, 2002). These authors also stated that oxidative damage was not directly involved in the impairment of photosynthetic machinery of plants under waterlogged conditions. In contrast, increase in the activities of different enzymatic antioxidants – SOD, CAT, peroxidase (POD) and APX – was recorded in pigeon pea genotypes when subjected to varying degrees of waterlogging stress (Kumutha *et al.*, 2009).

El-Enany *et al.* (2013) carried out a pot experiment with three replicates of 75% and 50% water deficit (WD) and one-fold field capacity waterlogging (WL) on cowpea (*Vigna sinensis*) plants. The data revealed that both stresses significantly decreased the fresh and dry weights of roots and shoots, number of nodules per plant and nitrogenase activity. Antioxidant metabolites like phenolic compounds, ascorbic acids, proline, MDA and H_2O_2 were significantly increased under WD and WL. The activities of certain antioxidant enzymes (SOD, CAT and APX) under both stresses were determined (El-Enany *et al.*, 2013).

Waterlogging reduces the endogenous levels of nutrients in different parts of the plant (Ashraf *et al.*, 2010, 2012). Oxygen deficiency in the root zone causes a marked decline in the selectivity of K^+/Na^+ uptake and impedes the transport of K^+ to the shoots (Ashraf *et al.*, 2012). When *Medicago sativa* was subjected to flooding stress, a marked reduction in leaf and root nutrient composition (P, K, Ca, Mg, B, Cu and Zn) was recorded in plants (Smethurst *et al.*, 2005). Among cool season

food legumes, faba bean is more tolerant to waterlogging than lentil, pea and chickpea (Siddique, 2000).

Many management practices used to reduce the effects of waterlogging involve paddock selection, sowing time, seeding rate and drainage (Toker & Mutlu, 2011). Genetic variation in waterlogging tolerance in food legumes deserves attention (Toker *et al.*, 2007a; Toker & Mutlu, 2011).

1.2.4 Legumes under temperature extremes

Temperature is one of the major factors affecting the yield and quality of legumes (Christophe *et al.*, 2011). Heat stress often is defined as high temperatures that cause irreversible damage to plant function or development after a certain period of exposure (Bhattacharya & Vijaylaxmi 2010; Hasanuzzaman *et al.*, 2013). Plants can be damaged in different ways by either high day or high night temperatures, and by either high air or high soil temperatures. Also, crop species and cultivars differ in their sensitivity to high temperatures. High temperature may negatively affect photosynthesis, respiration, water relations and membrane stability, and also modulate levels of hormones and primary and secondary metabolites. Furthermore, throughout plant ontogeny, enhanced expression of a variety of heat-shock proteins, other stress-related proteins, and production of ROS constitute major plant responses to heat stress (Bhattacharya & Vijaylaxmi, 2010; Hasanuzzaman *et al.*, 2013).

Cool-season annual species are more sensitive to hot weather than warm-season annuals (Hall, 2001). Heat stress affected nitrate assimilation in legumes by lowering synthesis of ureides and decreasing levels and activities of nitrate reductase and glutamate synthase (Hungria & Vargas, 2000; Christophe *et al.*, 2011). In nodules, heat stress may either affect nitrogenase activity leading to decreased N_2 fixation efficiency or accelerate nodule senescence resulting in reduced nodule longevity (Bordeleau & Prévost, 1994; Hungria & Vargas, 2000; Christophe *et al.*, 2011).

Chilling stress is usually limited to plants that are native to or growing in tropical or subtropical regions of the world. Plants vary greatly in their sensitivity to chilling stress. Chill-sensitive plants have been defined as plants that are killed or injured by temperatures up to 15–20°C above the freezing point of the tissues (Bhattacharya & Vijaylaxmi, 2010; Hasanuzzaman *et al.*, 2013). Chill-resistant plants can grow at

temperatures near 0°C (Bhattacharya & Vijaylaxmi, 2010; Hasanuzzaman *et al.*, 2013).

Oktem *et al.* (2008) investigated the effects of cold and drought stress on growth parameters and antioxidant responses in shoots and roots of lentil seedlings subjected to drought and cold (4°C) stress for 5 days. They reported that the length and fresh weight of shoots decreased significantly under both stress conditions, contrary to the increase in these growth parameters for roots under the same conditions. The increase in proline levels was more pronounced under cold stress in shoots and roots. The oxidative damage resulted in increase of malondialdehyde (MDA), and hydrogen peroxide (H₂O₂) was markedly higher in shoots under cold stress. Both stress conditions caused a significant increase in malondialdehyde levels in root tissues. SOD activity was differentially altered in shoot and root tissues under drought and cold stress. The CAT activity was higher in roots under drought stress, whereas APX activity increased in root tissues under cold stress (Oktem *et al.*, 2008; Bhattacharya & Vijaylaxmi, 2010).

1.2.5 Legumes under soil acidity

Approximately 40% of the world's arable land is considered to be acidic (Valentine *et al.*, 2011). In natural ecosystems, soil acidity determines the availability of mineral nutrients such as phosphorus (P) and also determines the level and severity of phytotoxic elements such as aluminium (Al), manganese (Mn) and iron (Fe) (Muthukumar *et al.*, 2014). Al ions present in acidic soils cause (i) induction of ROS and lipid peroxidation, which limit crop yield in these soils (Yamamoto *et al.*, 2002; Muthukumar *et al.*, 2014); and (ii) extrinsic toxicity through calcium (Ca) and magnesium (Mg) deficiency (Kinraide *et al.*, 2005; Muthukumar *et al.*, 2014).

Three possible groups of mechanisms appear to operate in plants that can tolerate acidic conditions (Muthukumar *et al.*, 2014). These include the following:

- 1 Exclusion of toxic ions such as Al and Mn from the root apex.
- 2 Tolerance to toxic levels of Al and Mn through detoxification in the plant symplasm.
- 3 Enhanced efficiency in the uptake of limiting nutrients from acid soils (Kochian *et al.*, 2004; Bhalerao & Prabhu, 2013).

Soil acidity is a major factor affecting the growth and yield of legumes in many of the world's agricultural systems, due to the effect of phosphorus (P) deficiency and

Al toxicity on SNF; it might be considered as a main limitation on the contribution of legumes to the global N cycle (Valentine *et al.*, 2011).

1.2.6 Legumes under nutrient deficiency

In agricultural soils, deficiency of some elements negatively affects nitrogen fixation in legumes and consequently reduces their yield. Toker and Mutlu (2011) reported that in chickpea, N and P deficiencies resulted in yield losses of 790,000 and 653,000t/year, respectively, worldwide. In most legume-growing soils, N and P are at either low or medium levels, whereas potassium (K) is usually sufficiently available to support growth, although it can be deficient in some soils (Srinivasarao *et al.*, 2003). Ca and Mg are generally deficient in acid soils (pH<5.5).

Sulphur (S) deficiency has been reported on light-textured soils in India, and the application of S at 20kg/ha is recommended for these soil types (Srinivasarao *et al.*, 2003). S deficiency is also seen in calcareous soils with a pH of 8.0 or higher (Toker *et al.*, 2011). Iron (Fe) deficiency has been recorded in many legume crops such as chickpea, lentil, lupin, pea, bean and soybean (Erskine *et al.*, 1993; Toker *et al.*, 2010).

Al toxicity induced reduction of SNF due to (i) the inhibition of rhizobial growth in the soil; (ii) the retardation of nodulation; and (iii) the possible alteration in organic acid metabolism (Valentine *et al.*, 2011). Boron (B) toxicity or deficiency induced suppression of normal growth in pea or faba bean (Dwivedi *et al.*, 1992; Poulain & Almohammad, 1995).

Toker and Mutlu (2011) reported that in legume species the relative sensitivity to zinc (Zn) deficiency is high for common bean relative to soybean (Alloway, 2009). Lentil, chickpea and pea were found to be more sensitive to Zn deficiency than oilseeds and cereals (Tiwari & Dwivedi, 1990). Differential Zn efficiency was reported among navy bean genotypes (Jolley & Brown, 1991; Moraghan & Grafton, 1999). Zn deficiency caused delay in pod maturity in bean (Blaylock, 1995).

1.3 Breeding of cool season food legumes

In 1970s, the breeding of legumes started with the establishment of the International Centre for Agricultural Research in Dry Areas (ICARDA) in Syria and the International Crops Research Institute for

Semi-Arid Tropics (ICRISAT) in India, supported by the Consultative Group in International Agricultural Research (CGIAR), as well as through strengthening of the agricultural research systems of different conditions (Materne *et al.*, 2011). Genetic resources for use in cool season food legume breeding are maintained at ICARDA, ICRISAT and also by other national programmes, particularly in the USA, Canada, Australia, India and a number of other important repositories (Materne *et al.*, 2011).

1.4 Breeding of cool season food legumes under abiotic stress

Legumes are considered more sensitive than cereals to a wide range of abiotic stresses (Dita *et al.*, 2006). Materne *et al.* (2011) reported that matching a crop's phenology to an environment, including the avoidance of drought and heat, is a key part of improving adaptation and increasing crop yields, and has been a major global focus in breeding for local and broad adaptation of all the cool season food legumes (Materne & Siddique, 2009; Khan *et al.*, 2010). One of the major achievements of ICARDA's collaborative lentil research is broadening the narrow genetic base of lentil in South Asia through introgression of genes from ICARDA germplasm (Materne *et al.*, 2011).

In short growing season climates (both winter- and spring-sown crops), selection for earlier flowering genotypes has been an important trait for avoidance of late season abiotic stress (e.g. terminal drought and high temperatures) (Materne *et al.*, 2011). In chickpea, whilst the *Kabuli* type is generally considered more drought sensitive than *Desi* types (Leport *et al.*, 2006), ICRISAT developed an extra-short-duration *Kabuli* variety (ICCV 2), which improved yields and expanded production. Since the release of this cultivar, even earlier-maturing germplasm has been developed and combined with a double-podding trait (Ahmad *et al.*, 2005).

Salinity is attracting greater attention from researchers and breeding programmes internationally (Materne *et al.*, 2011). Lentil cultivars with improved tolerance to NaCl have been released already in Australia (Materne & Siddique, 2009). Flowers *et al.* (2010) give a comprehensive overview of studies conducted to explore genetic variation to salt sensitivity in chickpea. Greater efforts have also been focused on quantifying thresholds, and it

was recently reported that subsoil chloride (Cl) concentration was the most effective indicator of reduced grain yields rather than salinity, and that growing chickpea on soils with Cl > 600 mg/kg should be avoided due to high yield losses (Dang *et al.*, 2010). Similarly, faba bean has been reported to be more sensitive to Cl than Na, and genetic variation for tolerance to the individual ions was observed (Tavakkoli *et al.*, 2010). Screening methodologies range from pot-based to field methods. More recently, attention has been focused on improving genetic knowledge that could provide molecular markers for salt tolerance in the near future (Varshney *et al.*, 2009).

Cold tolerance has been an important trait for improvement in crop adaptation in many countries (Materne *et al.*, 2011). For chickpea, chilling temperatures at the reproductive phase often result in pod abortion. Clarke *et al.* (2004) successfully used pollen selection methods to develop and release two cultivars that produce pods under lower temperatures than other cultivars. In the USA and Turkey, large yield increases have been achieved by sowing lentil in winter rather than spring, using genotypes tolerant to cold temperatures during winter (Materne & McNeil, 2007). Similarly, very high tolerance of seedlings to cold temperatures has been identified in faba bean (Link *et al.*, 2010). In Australia, lentil breeding lines with improved tolerance to boron have been developed that could improve yields by up to 91% in the target region, based on controlled environment experiments (Hobson *et al.*, 2006). Whilst genetic variation has been identified in chickpea (Hobson *et al.*, 2009), only limited research in this crop has been undertaken. Genetic variation has been identified in field pea (Redden *et al.*, 2005), and the overall level of tolerance of this crop is greater than in lentil and chickpea (Materne *et al.*, 2011).

1.5 Breeding of warm season food legumes

Singh *et al.* (2011) reported that efforts focused on the breeding of warm season food legumes have been made in different international centres supported by CGIAR. Among these centres, ICRISAT has focused research on pigeon pea, and the International Institute of Tropical Agriculture (IITA) has a global mandate for cowpea improvement. The Asian Vegetable Research and

Development Centre (AVRDC) was established for the improvement of mung bean worldwide. Besides, the US Department of Agriculture (USDA) has focused research activities on soybean. The Indian Institute of Pulses Research, Kanpur, a leading centre of the Indian Council of Agriculture Research, and other Agriculture Universities in India are also involved in genetic improvements in warm season legume crops, including pigeon pea, mung bean and urd bean. These national and international centres are involved in collection, evaluation and sharing of germplasm, and also undertake breeding programmes for genetic improvement. The international centres also distribute the segregating populations and inbred lines to partner countries for selection and release as varieties, resulting in stimulation of breeding internationally. Hall *et al.* (1997) and Singh *et al.* (1997) have described cowpea breeding programmes in different regions of the world. The bean/cowpea CRSP (Cowpea Collaborative Research Program) is also catalysing and supporting research on cowpea improvement in the USA, Cameroon and Senegal. Significant research on various aspects of cowpea improvement is also being carried out in Brazil, Nigeria, Burkina Faso, Senegal, Mali and India and, to a lesser extent, in a number of other countries. These efforts have led to the development of different types of cowpea cultivar, including *Vigna unguiculata*, *Vigna biflora* (or catjang) and *Vigna sesquipedalis* (Hall *et al.*, 1997).

1.6 Breeding of warm season food legumes under abiotic stress

Singh *et al.* (2011) summarized some important target traits in breeding programmes for improving the warm season food legumes against abiotic stress, as detailed below.

1.6.1 Short duration and photo-thermal insensitivity

These are important traits in soybean, mung bean and urd bean, because the development of short-duration and photo-thermally insensitive genotypes creates plants suitable for different cropping systems, and also avoids terminal drought (Singh *et al.*, 2011). In cowpea, photosensitive cultivars not only flower early but also become extremely dwarf in habit when day length is under 12.5h (Ishiyaku & Singh, 2001), and a complete

association of photosensitivity has been observed with dwarfing, which is controlled by a single recessive gene (Ishiyaku & Singh, 2001). In urd bean, earliness and photo-thermosensitivity are recessive traits and are controlled by major genes (Sinha, 1988). Thus selection of genotypes with early vigour holds tremendous importance in breeding programmes. As a result, some of the very popular early varieties, such as Narendra Urd 1, KU 300, Sarla, Vamban and Urd 3, have been developed in India for commercial cultivation. Since urd bean is also cultivated in the spring/summer season, Pant U 19, T 9, KM 1 and TMV 1 have been developed as photo-thermoinsensitive varieties (Gupta & Kumar, 2006).

1.6.2 Leaf pubescence density

Suitability for soybean cultivation is improved by this trait in drought-prone areas, as it reduces leaf temperature and water loss by transpiration and enhances photosynthesis and vegetative vigour (Du *et al.*, 2009). Two additive genes control this trait in soybean (Pfeiffer & Pilcher, 2006). This is also an important trait of mung bean and urd bean; some lines of mung bean developed at AVRDC, e.g. V 2013, V 1281, V 3372, VC 1163D, VC 2750A, VC 2754A and VC 2768A, can withstand moisture stress (Tickoo *et al.*, 2006), including long spells of rainfall causing flooding.

1.6.3 Seed dormancy

Reduced seed dormancy is found in mung bean, resulting in preharvest sprouting during the maturity phase in the monsoon (*kharif*) season. Therefore the identification of lines with tolerance to preharvest sprouting is highly desirable, both in this crop (Tickoo *et al.*, 2006) and in urd bean.

1.6.4 Deep root system

Many desert plants have been reported not to have a deep root system, whereas deep rooting becomes more common in less extreme dry areas (Vadez *et al.*, 2007). In fact, the importance of any aspect of rooting pattern (depth, depth distribution, root length density, etc.) is totally relative to the distribution and amounts of water or nutrients in the soil profile. For example, increased root depth/root volume is useful only where there is significant water available to exploit by increasing the soil volume explored by the roots (Vadez *et al.*, 2007).

Kashiwagi *et al.* (2006) showed the importance of roots for seed yield under terminal drought conditions

in chickpea. It has been reported that chickpea was able to allocate more roots to the deeper soil layers under conditions of stress than other legumes (Benjamin & Nielsen, 2005), or than more sensitive genotypes (Kashiwagi *et al.*, 2006). However, this was so only when the phenology of the genotype was well suited to the test environment. For example, the chickpea genotypes K1189 and ICC898 had adequate root length density (RLD) compared to ICC4958 (Vadez *et al.*, 2007).

It is assumed that pigeon pea is deep-rooted and that confers drought tolerance because the crop is usually grown on deep soils and completes its life cycle on residual moisture (Vadez *et al.*, 2007).

1.7 Biotechnology approaches

Plant biotechnology offers new ideas and techniques applicable to agriculture. It uses the conceptual framework and technical approaches of plant tissue culture and molecular biology to develop commercial processes and products (Sharma & Lavanya, 2002). These techniques enable the selection of successful genotypes, better isolation and cloning of favourable traits, and the creation of transgenic crops of importance to agriculture. This ability has moved agriculture from a resource-based to a science-based industry (Sharma & Ortiz, 2000; Sharma & Lavanya, 2002).

Legumes can face the threat posed by abiotic stress through several genetic improvement strategies, from classical breeding to more direct physiological genetic approaches.

1.7.1 MicroRNAs

MicroRNAs (miRNAs) play major roles in plant growth and development (Jones-Rhoades *et al.*, 2006; Khraiwesh *et al.*, 2012; Mantri *et al.*, 2013) as well as in regulating the synthesis of polypeptides from different mRNAs including those that act as transcription factors (Mantri *et al.*, 2013). They help plants to thrive under abiotic stresses such as drought, salinity and high/low temperature by regulating the expression of thousands of genes (Mantri *et al.*, 2013).

Next Generation Sequencing (NGS) technology has greatly accelerated the discovery and characterization of miRNAs in a range of diverse plant species (Sunkar & Jagadeeswaran, 2008; Zhao *et al.*, 2010; Chen *et al.*, 2011; Mantri *et al.*, 2013). The first isolation for miRNAs

was in *Arabidopsis thaliana* (Park *et al.*, 2002; Reinhart & Bartel, 2002). Since then they have been isolated from a wide range of species via genetic screening (Lee *et al.*, 1993; Wightman *et al.*, 1993), direct cloning after isolation of small RNAs (Fu *et al.*, 2005; Lu *et al.*, 2005) and computational prediction strategies (Wang *et al.*, 2005; Jones-Rhoades *et al.*, 2006).

Mantri *et al.* (2013) in their review reported that miRNAs play an important role in drought tolerance. These include conserved miRNAs such as miR164, miR169, miR171, miR396, miR398, miR399, miR408 and miR2118 (Liu *et al.*, 2008). Their expression patterns vary with legumes species. For example, miR169 was downregulated in *M. truncatula* (Trindade *et al.*, 2010) but upregulated in common bean (in response to abscisic acid treatment) (Zhao *et al.*, 2009). In *M. truncatula*, miR398a,b and miR408 were strongly upregulated in shoots and roots under drought stress (Trindade *et al.*, 2010). The miR398 and miR408 repress the *COX5b*, *CSD1* and plantacyanin genes (Trindade *et al.*, 2010). Under drought and ABA treatments, Arenas-Huertero *et al.* (2009) identified a number of novel legume miRNAs in *Phaseolus vulgaris*. Among them pvu-miRS1, pvu miR1514a, miR159.2, pvu-miR2118 and pvu-miR2119 accumulated upon drought and ABA treatments. Novel miRNAs may target regulatory elements for cellular processes that may be unique to legumes (Arenas-Huertero *et al.*, 2009). Forty-four drought-associated miRNAs (30 were upregulated in drought conditions and 14 were downregulated) were identified in cowpea under drought stress (Barrera-Figueroa *et al.*, 2011). Wang *et al.* (2011) in their study on *M. truncatula* subjected to drought stress, identified 22 members of four miRNA families that were upregulated and 10 members of six miRNA families that were downregulated. Among the 29 new miRNAs/new members of known miRNA families, eight miRNAs were responsive to drought stress of which four each were upregulated and downregulated. The drought-responsive miRNAs were found to be involved in diverse cellular processes including development, transcription, protein degradation, detoxification, nutrient status and cross-adaptation (Mantri *et al.*, 2013).

Mantri *et al.* (2013) in their review reported that legumes express a variety of miRNAs in response to salt stress. In cowpea under salinity stress, Paul *et al.* (2011) identified 18 conserved miRNAs belonging to 16 miRNA families. Fifteen miRNAs were predicted and identified

as transcription factors. Seven of these predicted miRNAs (vun-miR156a, vun-miR159b, vun-miR160a, vun-miR162a, vun-miR168a, vun-miR169b and vun-miR408) were experimentally validated in the root tissues and found to be upregulated during salt stress as revealed by qRT-PCR (Paul *et al.*, 2011). Dong *et al.* (2013) studied the dynamic regulation of miRNA in functioning soybean mature nodules under salt stress. The authors identified 110 known miRNAs belonging to 61 miRNA families, and 128 novel miRNAs belonging to 64 miRNA families. Among them, 104 miRNAs were dramatically differentially expressed (>2-fold or detected only in one library) during salt stress. The miR159bc, miR169c and miR319a,b were highly down-regulated and gly_1, gly_3, miR171p and miR4416d were highly upregulated by salt (Dong *et al.*, 2013; Mantri *et al.*, 2013). Recently, Nageshbabu and Jyothi (2013) analysed the expression of nine different miRNAs in *Phaseolus vulgaris* seedlings in response to 0.4M NaCl and drought stress. They reported that miR395 was most sensitive to both stresses and was upregulated under both the stressors. Further, miR396 and miR172 were upregulated after exposure to both the stresses (Mantri *et al.*, 2013; Nageshbabu & Jyothi, 2013).

Wang and Long (2010) by using RT-PCR showed miRNAs associated with cold tolerance in pea (*Pisum sativum*), and the level of their expressions increased after the cold treatment.

MicroRNAs are important signalling and regulatory factors in P deficiency stress (Mantri *et al.*, 2013). Under P starvation stress, miR399 was induced in common bean and *M. truncatula* (Valdes-Lopez & Hernandez, 2008). This miRNA plays a key role in maintaining Pi (inorganic phosphate) homeostasis in *Arabidopsis* and is induced under P deficiency causing repression of the ubiquitin conjugating enzyme UBC24, a repressor of phosphate transporters (Chiou *et al.*, 2006; Mantri *et al.*, 2013).

Zeng *et al.* (2010) identified 57 miRNAs under P deficiency in soybean. Also Sha *et al.* (2012), by using deep sequencing of soybean root and shoot libraries constructed under P stress, identified 60 known and conserved responsive miRNAs, belonging to 35 families. Also, 16 novel predicted miRNAs were identified. In a larger study, 167 miRNAs, belonging to 35 families, were identified via differential expression in response to P deficiency in white lupin; 17, 9 and 10 were found to be upregulated, while 7, 6 and 12 were downregulated in roots, stems and leaves, respectively (Zhu *et al.*, 2010).

Recently, Xu *et al.* (2013) showed that 25 miRNAs were induced and 11 mRNAs were repressed under P deficiency in soybean.

Sulphur deficiency induced the suppression of mRNA395 in legume species (Szittyta *et al.*, 2008; Kawashima *et al.* 2009). This mRNA regulates ATP sulphurylase (APS4) and a sulphate transporter (AST68) when maintaining S homeostasis during S deficiency (Mantri *et al.*, 2013).

Zeng *et al.* (2012) identified 30 stress-responsive miRNAs in Al-treated and non-treated roots. Of these, 10 were conserved miRNAs that belonged to seven families, 13 were unconserved and seven were novel. In soybean, miR396, miR390 and miR1510a-p5 were upregulated; miR156, miR164 and miR169 were down-regulated; and miR1510a was non-responsive to Al (Zeng *et al.*, 2012). Chen *et al.* (2012) identified several *M. truncatula* miRNA (miR160, miR319, miR396, miR1507 miR1510a and miR390) as down-regulated and other two (miR166 and miR171) not responsive to Al treatment. Using a computational approach, Zhou *et al.* (2008) identified 26 new miRNA candidates including miR160, miR166, miR319, miR393 and miR398 that were responsive to mercury, cadmium and aluminium stresses. Their differential expressions were subsequently assessed in various *M. truncatula* organs and tissues (Mantri *et al.*, 2013).

1.7.2 Molecular marker-assisted breeding

Molecular markers are DNA regions tightly linked to agronomic traits in crops, identified by using genetic and genomic analysis. They can facilitate breeding strategies for crop improvement. However, the use of molecular markers in breeding programmes needs preliminary studies to identify and validate potential markers (Dita *et al.*, 2006).

Several molecular marker-related techniques, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) and derivatives, have been reported for abiotic stresses (Kassem *et al.*, 2004; Lee *et al.*, 2004). This has enhanced knowledge of the genetic control of specific resistance and/or tolerance in many legumes by giving information on the number, chromosomal location and individual or interactive effects of the different quantitative trait loci (QTLs) involved (Dita *et al.*, 2006).

These technologies have identified specific molecular markers that may be used in breeding programmes via marker-assisted selection (MAS) to improve stress tolerance (Dita *et al.*, 2006). In legumes, the practical application of MAS for the genetic enhancement of resistance or tolerance to stress has been limited. Schneider *et al.* (1997) reported that MAS may be useful to select drought-tolerant common bean.

1.7.3 Gene pyramiding assisted by MAS

Pyramiding different resistance or tolerance traits into a genotype helps plant breeders to achieve resistance to abiotic stress. In legumes there are numerous examples of introgression and pyramiding of favorable alleles and QTLs. However, MAS has been used to help in gene pyramiding to overcome stresses in only a few cases (Dita *et al.*, 2006). Nevertheless, Schneider *et al.* (1997) indicated that MAS may be useful to select drought-tolerant common bean.

There are some exceptions where MAS has facilitated breeding efforts in several legume crops to combat important biotic stressors (Dita *et al.*, 2006). For example, MAS was successfully used for the breeding of soybean resistant to cyst nematode (Diers, 2004), of pinto bean resistant to common bacterial blight (Mutlu *et al.*, 2005) and of narrow-leaved lupin (*Lupinus angustifolius* L.) resistant to phomopsis stem blight (Yang *et al.*, 2002) and anthracnose (Yang *et al.*, 2004). Moreover, when resistance is conferred by single genes and/or easily overcome by new pathogen races, the gene pyramiding strategy facilitated by MAS can be an efficient method (Dita *et al.*, 2006).

Legume cultivars having appropriate combinations of resistance and/or tolerance to biotic and abiotic stresses, achieved through gene pyramiding, could provide durable resistance, and MAS can be a valuable tool to guide and identify the pyramiding of these genes (Dita *et al.*, 2006).

The generation of markers based on genes with altered expression patterns in response to stresses could result in more effective and targeted MAS. Some of these genes may be good candidates for future MAS studies in legumes (Dita *et al.*, 2006).

1.7.4 Somaclonal variation and *in vitro* mutagenesis

Tissue culture provides a big range of genetic variation in plants, which can be incorporated in plant breeding programmes (Jain, 2001; Dita *et al.*, 2006). Somaclonal

variation, including callus cultivation and somatic embryogenesis, has the ability to generate genetic variation (Larkin & Scowcroft, 1981; Dita *et al.*, 2006). The ability to produce agronomically useful somaclones via organogenesis and somatic embryogenesis has been reported in pea (Griga *et al.*, 1995) and pigeon pea (Chintapalli *et al.*, 1997).

Indeed, combining mutagenesis techniques with MAS through TILLING (see Section 1.7.9) will make mutagenesis more suitable for legume enhancement. The main problem with these techniques is the high quantity of individuals required to find the desired trait. Nevertheless, by using *in vitro* selection systems this disadvantage can be reduced (Dita *et al.*, 2006).

1.7.5 *In vitro* selection

In vitro selection is one of the important classical breeding methods (Svabova & Lebeda 2005) and has been used for both biotic and abiotic stresses. In legumes, *in vitro* selection was applied to alfalfa (*Medicago sativa*) for selection of resistance to *Colletotrichum trifolii* (Cucuzza & Kao, 1986), *Fusarium oxysporum* (Cvikrova *et al.*, 1992) and *Verticillium albo-atrum* (Koike & Nanbu, 1997). However, no resistant lines were reported in these studies (Dita *et al.*, 2006).

Putative stress-resistant lines derived from both conventional breeding and transgenic approaches could be screened using *in vitro* selection. This is suitable for some abiotic stresses, where appropriate screening methods are unavailable or have low efficiency. Somaclonal variation and *in vitro* mutagenesis followed by *in vitro* selection offer an alternative way for breeding (Dita *et al.*, 2006).

1.7.6 Transcriptomics

Repression of genes or transcriptional activation is an important tool in the control of stress responses in plants (Chen *et al.*, 2002; Dita *et al.*, 2006). Thus, identification of differentially expressed genes is particularly important to understand stress responses in plants. To achieve this objective, tools such as microarrays (Schena *et al.*, 1995), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), suppression subtractive hybridization library (Diatchenko *et al.*, 1996), and quantitative measurement of transcription factor (TF) expression have been developed in addition to older techniques such as Northern blotting (Dita *et al.*, 2006).

In legumes, transcriptomic techniques are useful ways of breeding to combat environmental stresses. Jain

et al. (2001) reported 43 drought-responsive mRNA transcripts differentially expressed in peanut under water stress. Pnueli *et al.* (2002) applied suppression subtractive hybridization screening (SSHS) in *Retama raetam*, a C₃ drought-tolerant legume. The authors revealed that dormancy, key to the survival of many species in arid environments, was followed by accumulation of transcripts encoding PR-10-like protein, a low-temperature-inducible dehydrin and a WRKY transcription factor. Umezawa *et al.* (2002), by using a modified c-DNA-AFLP technique in soybean, revealed 140 differentially expressed cDNA fragments by comparing control and iso-osmotic treated plants. Some of the responsive genes encoded ion transporters, transcription factors (TFs) and redox enzymes (Dita *et al.*, 2006). From the *Arabidopsis* database, Ishitani *et al.* (2004) selected 100–200 genes, and revealed that at least three *DREB*-like genes might be key transcriptional regulators of drought and/or cold resistance in common bean (Dita *et al.*, 2006).

1.7.7 Proteomics

A proteomics approach is used to investigate the pathways of biochemical activities and the different responses of plants to stress (Aghaei & Komatsu, 2013). Plant stress proteomics has the ability to identify possible candidate genes that can be used for the genetic enhancement of plants against stresses (Cushman & Bohnert, 2000; Ngara, 2009; Rodziewicz *et al.*, 2014).

In legumes, proteomic techniques have been applied to cowpea, pea and lupin for identification of proteins involved in responses to different abiotic stresses (Fecht-Christoffers *et al.*, 2003; Repetto *et al.*, 2003; Kav *et al.*, 2004; Pinheiro *et al.*, 2005; Cheng *et al.*, 2010).

Proteomic analysis of *M. truncatula* under drought stress reported that the decline in SS is one of the most observable changes in plant function in root nodules (Larrainzar *et al.*, 2007). However, a plant system approach including the proteome and metabolome responses of *M. truncatula* nodules to drought revealed that the decline in SS was not correlated with a decrease in malate concentration (Larrainzar *et al.*, 2009), in contrast to studies of nodules of grain legumes (González *et al.*, 2001; Gálvez *et al.*, 2005). This relatively contrasting behaviour of *Medicago* species to other legumes is intriguing (Arrese-Igor *et al.*, 2011). One theoretical explanation is that in pasture legumes such as *Medicago*, grazing has produced a strong evolutionary selective

pressure on continued nodular SS activity during drought stress (Arrese-Igor *et al.*, 2011).

Proteome analysis of *M. truncatula* nodules provided good evidence of drought stress affecting the enzyme Met synthetase (Larrainzar *et al.*, 2007). Depressed Met availability had a major effect on both protein synthesis and sulphur metabolism in nodules (Arrese-Igor *et al.*, 2011).

Aghaei *et al.* (2009) and Sobhanian *et al.* (2010) studied the proteome of soybean under salt stress by using different tissues. They identified a 50S ribosome protein that was downregulated in leaves. Alam *et al.* (2010) studied the proteome analysis of soybean root under water stress. They indicated that two key enzymes involved in sugar metabolism, UDP-glucose pyrophosphorylase and 2,3-biophosphoglycerate-independent phosphoglycerate mutase, were downregulated under drought stress.

Cheng *et al.* (2010) reported 40 proteins (25 upregulated and 15 downregulated) in soybean seeds exposed to cold stress (4°C). These proteins are involved in cell growth/division, storage, cellular defences, energy protein synthesis, transcription and transport. Zhu *et al.* (2006) reported that the activation of HSP70 in transgenic lines by its upstream gene *HsfA1* improved soybean tolerance under high temperature stress.

1.7.8 Transgenomics

Transgenic technology is one of the many tools available for modern plant improvement programmes (Jewell *et al.*, 2010). The use of transgenic approaches, or 'transgenomics', helps in understanding the mechanisms governing stress tolerance, providing good ways for the genetic enhancement of field crops, thereby alleviating some of the major constraints to crop productivity in developing countries (Sharma & Ortiz 2000; Reddy *et al.*, 2012). Transgenic plants or their germplasm can be used as sources of new cultivars or as new sources of variation in breeding programmes (Jewell *et al.*, 2010).

When plants are subjected to abiotic stresses, a number of genes are turned on causing increased levels of several osmolytes and proteins that may be responsible for conferring a certain degree of protection from these stresses. Thus, it may be necessary to transfer several potentially useful genes into the same plant in order to obtain a high degree of tolerance to drought or salt stress (Reddy *et al.*, 2012).

There are several transgenic technologies for improving stress tolerance involving the expression of functional genes (Reddy *et al.*, 2012), including those

encoding enzymes required for the biosynthesis of osmoprotectants or late embryogenesis proteins, detoxification enzymes and modification of membrane lipids (Ishizaki-Nishizawa *et al.*, 1996; McKersie *et al.*, 1996; Xu *et al.*, 1996; Hayashi *et al.*, 1997; Bhatnagar-Mathur *et al.*, 2008; Reddy *et al.*, 2012; Rodziewicz *et al.*, 2014).

Osmotic adjustment (osmotic regulation) is one of the useful mechanisms for improving abiotic stress tolerance, especially if osmoregulatory genes can be triggered in response to drought, salinity and high temperature (Reddy *et al.*, 2012). Sharmila *et al.* (2009) showed that H₂O₂ produced by a prokaryotic osmoregulatory choline oxidase gene (*codA*) as a by-product during synthesis of glycine-betaine is responsible for building a stronger antioxidant system in chloroplasts of transgenic chickpea plants. Similarly, at ICRISAT, the P5CSF129A gene encoding the mutagenized D1-pyrroline-5-carboxylate synthetase (P5CS) for the overproduction of proline was introduced in chickpea. In several of these transgenic events the accumulation of proline in leaves increased significantly when the plants were exposed to water stress, along with a decrease in free radicals as measured by a decrease in the MDA levels, a lipid peroxidation product (Reddy *et al.*, 2012).

To date, genetic transformation has been reported in all the major legume crops such as *Vigna* spp., *C. arietinum*, *C. cajan*, *Phaseolus* spp., *Lupinus* spp., *Vicia* spp., *P. sativum*, soybean, groundnut, pigeon pea and chickpea (Sharma & Lavanya, 2002; Reddy *et al.*, 2012).

1.7.9 Targeting induced local lesions in genomes (TILLING)

Recently, Kudapa *et al.* (2013) in their review reported that validation of genes through genetic transformation, RNAi or virus-induced gene silencing (VIGS) is a time-consuming process in legumes, mainly due to lack of efficient transformation systems in legumes. This situation has promoted the application of TILLING to study gene function. In TILLING, candidate genes are screened across a mutant population (with point mutations), and line(s) with the mutation for the target gene are identified (McCallum *et al.*, 2000). If the identified line exhibits the expected phenotype for the candidate gene, the function of the candidate gene is supported. The TILLING approach could be preferred over RNAi for irreversibly inhibiting or eliminating the target genes in commercial crop plants since it avoids genetic transformation and increases stability of the phenotype (Barkley & Wang, 2008).

TILLING populations have been developed for several legumes. For example, in the model legumes *Medicago* (12,000 M2 plants; Rogers *et al.*, 2009) and *Lotus* (4904 M2 lines; Perry *et al.*, 2009) mutant populations were developed for use in reverse genetics. In the case of crop legumes, over 3000 M3 lines were developed in common bean and evaluated with root nodulation tests by Porch *et al.* (2009). In peanut a TILLING population of 10,000 lines has been established, and a subset of this population investigated for allergenicity (Tadege *et al.*, 2009). In chickpea, a TILLING population of ~3500 lines has been developed and is being used to identify candidate genes for drought tolerance (M. Thudi, personal communication). The use of NGS technologies for TILLING may increase the application of TILLING in crop legumes (Kudapa *et al.*, 2013).

EcoTILLING is a variant of TILLING, except that its objective is to discover naturally occurring polymorphisms as opposed to experimentally induced mutations (Kudapa *et al.*, 2013). Single nucleotide polymorphisms (SNPs), small insertions and deletions, and variations in microsatellite repeat number can be efficiently detected using the EcoTILLING technique (Kudapa *et al.*, 2013). For example, in legumes this method has been used to develop molecular markers for cyst nematode candidate resistance genes in soybean (Liu *et al.*, 2012). In mungbean it has been proven to be a valuable method for detecting polymorphisms in a collection that was previously shown to have limited diversity (Barkley & Wang, 2008).

1.8 Conclusions and future prospects

Food legumes are affected by abiotic stresses like salinity, water stress (drought and waterlogging), extreme temperatures (heat and cold) and nutrient deficiency, which ultimately lead to huge economic losses globally. Like other plant species, the breeding process in food legumes consists of four stages: (i) creating variations with hybridizations and induced mutations; (ii) selection in early generations; (iii) evaluation of selected lines; and (iv) release of varieties (Toker & Mutlu, 2011).

The biotechnological approaches of resistance breeding have provided several improved varieties of food legumes with tolerance to abiotic stresses. There is no substitute for these approaches, and they will continue to be the mainstay in the future. However, efforts

are needed to improve the effectiveness of these approaches by further refining screening methods for resistance to stresses and identifying new sources of resistance genes in both cultivated and wild species. There is a need to use diverse sources of resistance in breeding programmes and to develop cultivars with tolerance to multiple stress factors.

Mutagenesis facilitates an increase in genetic variability for resistance to abiotic stresses in food legumes. Transgenic legumes provide a great chance, but genes can flow from transgenics to wild relatives, leading to environmental pollution when transgenics are grown in the areas where wild relatives exist.

Modern techniques including all the 'omics', such as proteomics, genomics, transcriptomics and metabolomics, will be helpful to study legume responses to abiotic stresses. However, successful application of 'omics' to abiotic constraints needs knowledge of stress responses at the molecular level, which includes gene expression to protein or metabolite and its phenotypic effects. Therefore, research dealing with other techniques such as MAS or even classical breeding will be able to take advantage of the results obtained from these 'omics' technologies.

Based on the above-mentioned information we can conclude that the support of biotechnology approaches to conventional breeding methods would lead to advancement in the development of improved cultivars of food legumes with tolerance to abiotic stresses.

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