Zn-use efficiency for optimization of symbiotic nitrogen fixation in chickpea (*Cicer arietinum* L.)

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Abstract: Zn deficiency is widespread in traditional areas of chickpea cultivation worldwide. It limits chickpea productivity and causes significant losses to the economies of the world's largest chickpea exporters. This review may be of interest to researchers who would like to contribute to the improvement of chickpea cultivation on Zn-depleted soils in an environmentally sustainable manner, namely via identification of genotypes with superior symbiotic performance under Zn-limited conditions. The primary aim of the current work is to familiarize the readers with the biology and symbiotic characteristics of chickpea, and also to provide the necessary background on Zn as an essential nutrient for symbiotic nitrogen fixation (SNF). Special attention has been paid to the choice of rhizobial strains compatible with chickpea. Strains that can serve as an inoculum for simultaneous analysis of many genetically diverse chickpea lines have been suggested. The genotypes listed in this work can be good starting material for identification of chickpea lineages useful for unraveling the molecular basis of Zn-use efficiency, SNF efficiency, or both.

Key words: Chickpea, *Cicer arietinum*, symbiotic nitrogen fixation, nodulation, rhizobia, zinc, micronutrient deficiency

1. Introduction

Leguminous plants are capable of symbiotic nitrogen fixation (SNF) due to their ability to undergo endosymbiosis with soil bacteria called rhizobia. This process takes place in specialized structures that develop on legume roots and are known as root nodules (Udvardi and Poole, 2013). As a member of the legume family, chickpea (*Cicer arietinum* L.) contains up to 30.6% protein and is one of the most important dietary sources of protein for human consumption (Wood and Grusak, 2006). Data from the Food and Agriculture Organization (FAO) indicate that chickpea was grown in 58 countries in 2014. Its worldwide production increased from 8.4 million metric tons in 2005 to 14.2 million in 2014, which places this crop among the top five commercially grown pulses. South Asian countries, primarily India and Pakistan, have been the top producers of this crop during the last decade, followed by Turkey and Australia. Turkey used to be the second largest exporter of chickpea in the world a decade ago and is currently the top third exporter of chickpea seeds (53.6 thousand metric tons, 2013). At the same time, there has been a nearly twofold decrease in the production area of chickpea in Turkey within the last 20 years (http://faostat3.fao.org/home/E). This trend may have a negative effect on the self-sustainability of Turkish agriculture and

on its position in international trade. Therefore, efforts should be made to use chickpea genetic potential more completely, by means independent of fertilizer application. It is widely recognized that fertilizers, especially synthetic nitrogen (N)-rich substances, are enemies of the soil and water ecosystems (Crews and Peoples, 2004), and also of human health (Johnson et al., 2010). Besides the exceptionally high nutritional value of chickpea grains, chickpea considerably improves soil quality for subsequently planted crops as, for example, it reduces the occurrence of soil-borne pathogens (Felton et al., 1995). However, the main benefit that soil receives from chickpea comes through biologically fixed N, which may amount to up to 140–176 kg N per hectare annually (Rupela and Saxena, 1987; Saraf et al., 1998). For example, chickpea N-fixation rates of 23–97 kg N ha^{-1} serve as an equivalent of 60–70 kg fertilizer N ha^{-1} for maize (Bhatia et al., 2001). The yield of cereals can be increased by as much as 70% if planted after the chickpea harvest (Aslam et al., 2003). The availability of micronutrients, such as zinc (Zn), may limit normal plant growth and development. Up to one-third of cultivated soils worldwide are Zn-deficient (Cakmak et al., 2017). Particularly, soils in main chickpea-growing areas contain low amounts of available Zn. It has been reported that 48.5% of soils in India, 70% in Pakistan, and

80% in Turkey are deficient for Zn. In fact, Zn deficiency in crops is a major concern worldwide, especially for alkaline soils where Zn becomes unavailable (Broadley et al., 2007; Alloway, 2008). Globally, the occurrence of soil Zn deficiency coincides with Zn deficiency in humans (Cakmak et al., 2017). In Turkey, extremely low availability of Zn to plants severely affects the yield and nutritional value of staple grains, such as wheat, and is associated with numerous human health disorders. Up to 99% of Zn applied as a fertilizer may remain strongly bound to soil particles; hence, a mere increase in the Zn-fertilization rate can alleviate the problem only partially (Cakmak et al., 1999). Thus, the ability to use soil Zn more efficiently must be enhanced in crops via dedicated breeding efforts. Chickpea is more sensitive to Zn deficiency than many other crop species (Tiwari and Dwivedi, 1990; Brennan et al., 2001). Application of Zn improves SNF in chickpea by increasing the nodule number and nodule dry weight (Misra et al., 2002; Das et al., 2012). Natural ecotypes and breeding varieties of chickpea vary in their SNF (Gul et al., 2014) and Zn-use efficiency (Khan et al., 1998b). Unfortunately, primary selection of SNF-efficient genotypes revealed their higher sensitivity to fungal infections, such that the potential net benefit from their application in agriculture is low. Therefore, selection for chickpea lines with better SNF properties makes sense only in fungal-resistant genetic backgrounds (Khurana and Dudeja, 1996). While efforts have been made to isolate high-nodulating chickpea genotypes (Rupela, 1994, 1997; Khurana and Dudeja, 1996; Dudeja et al., 1997) and independently to select for higher resistance to various fungal pathogens (e.g., Pande et al., 2006; Rashid et al., 2014), no attention has been paid to identification of chickpea lines with superior symbiotic performance under conditions of low Zn availability. Once established, such lines could be recommended for regions of traditional chickpea production, most of which include Zn-deficient soils. The use of better-nodulating Zn-efficient chickpea varieties could result in higher profits to farmers and rural communities, and also in higher availability of N to plants sown in the same fields after chickpeas. If genes or groups of genes responsible for better SNF performance at low Zn conditions are known, they can be introduced into chickpea varieties having other valuable traits (yield, drought and cold tolerance, etc.). This can be done by conventional breeding methods in combination with marker-assisted selection. Thus, an understanding of genes that are differentially regulated in lines contrasting for SNF efficiency at low Zn supply is important and can be achieved, for instance, by transcriptional profiling of plants very sensitive and very resistant to low Zn in terms of SNF using next-generation sequencing of the whole sample RNA (RNAseq); see Wang et al. (2009) for a description of the method. Results of the RNAseq analysis may be combined with quantitative trait loci (QTL) mapping (Miles and Wayne, 2008) to narrow down groups of genes associated with efficient Zn use. This review provides background information useful for the initiation of screening for chickpea lines with higher N-fixation efficiency at low Zn conditions.

2. Zn in biological systems

2.1. Importance of Zn in cellular processes
Zn is an essential trace element for all forms of life on earth and is the second transitional metal most commonly found in organisms after iron (Broadley et al., 2007). The status of Zn is rather unique among micronutrients because it functions as a cofactor in enzymes of all six known classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Barak and Helmke, 1993). Although Zn does not change its redox state under physiological conditions, the protein-bound as well as free-ion Zn(II) being a biologically active form, its roles in living systems are diverse (Maret, 2013). In enzymes, the functions of Zn may be structural (appropriate protein folding), catalytic (direct participation in a reaction), and cocatalytic (catalytic, regulatory, and structural). Zn also has a structural role in the stabilization of nonenzymatic proteins. Zn-binding sites can be found in various types of macromolecules, including membrane lipids (membrane stability) and nucleic acids (control of transcription and RNA metabolism). In fact, the largest known group of Zn-containing proteins, zinc finger domain proteins, may exert their effect on transcription via a number of mechanisms, including chromatin modification, RNA metabolism, and protein–protein interactions (Broadley et al., 2007; Alloway, 2008). Finally, in certain tissue types of animals, Zn functions as a messenger molecule, similar to Ca^{2+} (Maret, 2013). Like all other essential nutrients, Zn becomes limiting for these cellular functions under conditions of low availability (Alloway, 2008), but also becomes highly toxic if present in excess, especially for soil microorganisms, including rhizobia (Chaudri et al., 2000; Broos et al., 2005). Unlike Zn deficiency, however, Zn toxicity is considered to be a less pressing problem for soil organisms, crops, and human nutrition, and is associated with industrial pollution and agricultural mismanagement rather than the natural environment (Alloway, 2008).

2.2. Physiological functions of Zn in plants
Zn deficiency is a major factor that limits the production of crops worldwide. Plant processes affected by low Zn availability include carbohydrate metabolism, membrane integrity, protein synthesis, auxin metabolism, and reproduction. Broadley et al. (2007) and Alloway (2008) have reviewed these aspects comprehensively. Carbohydrate metabolism may be affected by Zn deficiency due to impaired photosynthesis, formation and transport

of sucrose, and starch biosynthesis. Photosynthetic reactions depend on an adequate supply of Zn because of the presence of this metal in key photosynthetic enzymes, such as RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) and carbonic anhydrase (in C4 plants). Chloroplast structure and chlorophyll synthesis also suffer from the lack of Zn. Aldolase and sucrose synthase, which regulate sucrose synthesis, are sensitive to Zn deficiency. Likewise, starch grain formation is adversely affected by low Zn availability due to the requirement of Zn for the activity of starch synthase. Not only synthesis but also sucrose allocation becomes impaired in Zn-deficient plants, possibly due to compromised integrity of membranes. Intact biological membranes are stabilized by Zn through interaction with phospholipids and SH-groups (sulfhydryl groups) of membrane proteins. Membranes must also be protected from reactive oxygen species (ROS). Two enzymes, catalase and superoxide dismutase, that are required for this protection also depend on Zn availability. Protein synthesis is affected under Zn deficiency via impaired transcription and translation. Zn is essential for the activity of RNA polymerase and also for protection of ribosomal RNA from digestion by ribonuclease. The requirement of Zn for these processes, which are closely associated with intensive cell division, is thought to be the cause of high sensitivity of meristematic cells to Zn deficiency. Another important component of plant metabolism adversely influenced by the lack of Zn is the biosynthesis and possibly the stability of auxins, particularly indole acetic acid (IAA). Zn is required for the synthesis of tryptophan, which is a precursor of IAA (Broadley et al., 2007; Alloway, 2008). As a likely consequence of Zn involvement in ROS detoxification and its importance for membrane function and integrity, Zn application alleviates water stress, as shown also in

legumes such as alfalfa and chickpea (Khan et al., 2003, 2004; Grewal and Williams, 2008). In the model legume *Medicago truncatula*, Zn also increased plant resistance to fungal pathogen *Rhizoctonia solani* (Streeter et al., 2001). A similar effect on the severity of *Rhizoctonia* infection was observed in wheat (Thongbai et al., 2001). Zn availability may be influenced strongly by interaction with some macronutrients (P, N, Ca, Mg, K) and micronutrients (Fe, Cu, Mn, B, Na). The nature of these interactions is generally antagonistic and may be relatively simple, as in the case of N, which promotes vegetative growth and thus triggers a "dilution effect" for Zn concentrations within plant tissues. Interactions with other nutrients, such as P and Fe, may be quite complex. This aspect has been reviewed extensively by Alloway (2008), Hafeez et al. (2013), and authors referenced therein. Siddiqui et al. (2015) examined the interaction between Zn and P in chickpea. In this study, high levels of P application had an inhibitory effect on Zn uptake and translocation from roots to shoot, whereas a positive interaction between Zn and P was observed under P-deficient conditions. It was suggested that chickpea genotype IC269837 that accumulates high levels of Zn (see also Table 1) may be suitable for planting on soils with low P content.

2.3. Potential functions of Zn in SNF

There appears to be very little information available on the specific role of Zn in SNF, which is known to be promoted by adequate Zn supply (O'Hara et al., 1988; O'Hara, 2001). Initially, it was proposed that the observed improvements to SNF of Zn-deficient plants following Zn application were due to optimization of growth processes in the host plant rather than being a direct effect of Zn on N fixation (Lo and Reisenauer, 1968; Robson, 1978). Nodule number and size, leghemoglobin content, and the amount of N fixed were found to depend on Zn availability in soybean

Table 1. Chickpea genotypes tested for their tolerance to Zn deficiency.

#	More Zn-efficient genotypes	Less Zn-efficient genotypes	References
1	ICC-4958, T-1587, CTS-11308, NEC-138-2 × CM-72*, and Punjab-91	Tyson, CM-88*, Piadar-91, and C-44	Khan HUR (1998). Responses of chickpea (*Cicer arietinum* L.) to zinc supply and water deficits. PhD thesis. University of Adelaide.
2	CTS-60543, CTS-11308, and T-1587	Tyson and Dooen	Khan et al., 1998b
3	CM-88* and CM-31-1	6153, CM-72*, NIFA 95	Kausar et al., 2000
4	Barwon, ICC-4958, CTS-11308, and CTS-60543	Tyson and Dooen	Khan et al., 2000
5	G8 (IC269837), G20 (IC269817), G5 (IC269814)	G2 (1C269831), G14 (IC269867), G18 (IC269870), and G19 (IC269794)	Siddiqui et al., 2013
6	IC269837	IC269867	Siddiqui et al., 2015

* Note contradictory results for these two chickpea genotypes across different studies.

(Demeterio et al., 1972), cowpea (Marsh and Waters, 1985), and chickpea (Shukla and Yadav, 1982; Yadav and Shukla, 1983; Misra et al., 2002; Das et al., 2012). It was concluded from these studies that Zn is likely to be involved in leghemoglobin biosynthesis. Despite the lack of dedicated research on this subject, it is conceivable that, from the plant's perspective, the number of essential processes required for the establishment and function of SNF may be Zn-dependent. One such stage is early root nodule morphogenesis, which is controlled by auxin (Ferguson and Mathesius, 2014) and relies upon proper functioning of the apical meristem of the nodule (Łotocka et al., 2012; Franssen et al., 2015). Another apparently Zn-dependent stage (possibly affected via transcription and translation) occurs during the enlargement of the rhizobia-infected nodule cells (up to 80-fold). In *M. truncatula*, which is a taxonomically close relative of chickpea from the same Galegoid clade of the Papilionoideae legume subfamily (Varshney et al., 2009), this morphological change is accompanied by endoreduplication of the plant cell DNA (up to 64-fold compared to the haploid genome) to accommodate as many as 50,000 bacteroids per cell and to cope with very high levels of metabolism (Maróti and Kondorosi, 2014). The supply of photosynthetic carbon, primarily sucrose, to symbiotic bacteria inside the nodule is the main benefit that the microsymbiont receives from the association with the plant (Vance et al., 1998; Kryvoruchko et al., 2016). Long-distance transport of sucrose from leaves to the root nodules is very likely to be influenced by Zn availability, as a consequence of its dependence on intact membranes. As mentioned earlier, the synthesis of sucrose is also Zn-dependent. Finally, since rhizobia inside the root nodule are entirely surrounded by a plant-derived membrane (symbiosome membrane), they completely rely upon the export of all nutrients from the plant side (Udvardi and Poole, 2013). This transport across the symbiosome membrane requires high integrity of all its components, which is expected to depend on adequate Zn availability.

2.4. Zn is essential for optimal growth of rhizobia

Zn requirement as well as toxicity to symbiotic N-fixing bacteria was first demonstrated on five rhizobial strains by Wilson and Reisenauer (1970), who found that the sensitivity of the five tested organisms to Zn deficiency and Zn toxicity varied. The amount of Zn^{2+} initially required in batch culture for maximal growth was in the range of 0.1–1.0 µM, whereas 10 µM of Zn^{2+} was the concentration toxic to most of the strains, although to different extents (0.4%–49.0%). Complete absence of Zn^{2+} from the culture medium inhibited the culture growth by 1%–20% relative to the control (Wilson and Reisenauer, 1970). Later experiments with *Bradyrhizobium* spp. indicated that sensitivity to low Zn in low-cell-number batch culture was a strain-specific

rather than a species-specific character. Zn concentrations below the range of 1.0–100.0 nM were growth-inhibiting depending on the strain (O'Hara, 1988). So far, there is no report of Zn having a specific function in N fixation on the bacterial side. Still, the following considerations offered by Broadley et al. (2007) for a different prokaryotic organism (*Escherichia coli*) give an idea of how Zn deficiency may affect the settling of rhizobia within the root nodules. It appears that Zn inside an *E. coli* cell is present in negligible amounts in a soluble form (Zn^{2+} ion), while more than 10% of all Zn in this organism (out of an estimated 200,000 atoms per cell) is bound to just six proteins. The major of them is RNA polymerase that incorporates two Zn atoms per protein, but being expressed at a rate of 5000 copies per cell hosts 10,000 atoms of Zn in total. Five other proteins are tRNA synthases that bind one Zn atom per protein, but are present in the cell in 2000–3000 copies each (Outten and O'Halloran, 2001). There are at least 30 more proteins that carry tightly bound Zn (Katayama et al., 2002) and a number of other organic molecules with lower affinity to Zn (Outten and O'Halloran, 2001). Given the above, it seems reasonable to assume that transcription and translation are likely to be at least somewhat limited in a prokaryotic cell if the Zn supply is insufficient. Rhizobial symbionts within the root nodules of many legume species, including chickpea (Kantar et al., 2007; Montiel et al., 2016), undergo irreversible differentiation to become organelle-like structures, bacteroids. At early stages, this differentiation is characterized by very high rates of transcription and metabolism and relies on intensive cell division, multiple rounds of bacterial DNA endoreduplication (up to 24-fold), and profound changes to the prokaryotic cell morphology (Mergaert et al., 2006; Maróti and Kondorosi, 2014; Montiel et al., 2016). If such fundamental processes as RNA synthesis and protein synthesis lack Zn for their basic machinery, it may be difficult for rhizobial cells to make the transition to bacteroids, which is vital for their function in SNF.

2.5. Zn-use efficiency in plants and its relevance to SNF

2.5.1. Mechanisms of Zn-use efficiency

The efficiency of Zn use has been studied in cereals and grain legumes, including chickpea. Following Alloway (2008), we consider here the plant's tolerance to Zn deficiency as being synonymous to Zn-use efficiency. Significant differences in the efficiency of Zn utilization have been observed between faba beans, chickpea, wheat, and lentil (in decreasing order of efficiency; Brennan et al., 2001). At the same time, considerable intraspecific variation in this parameter has been reported in wheat, barley, oat (Graham et al., 1992), rice (Neue et al., 1998), and chickpea (Khan et al., 1998b, 2000; Kausar et al., 2000; Siddiqui et al., 2013). Possible mechanisms of Zn efficiency have been extensively discussed by Alloway (2008).

The author refers to the summary on such mechanisms proposed by Rengel (1999): 1) root architecture better suited for more complete access to soil pockets (a greater proportion of longer, thin roots); 2) chemical properties of the rhizosphere, including more intensive secretion of Zn-chelating agents (phytosiderophores or, to be more accurate, phytometallophores), and activity/diversity of soil microorganisms; 3) more intensive acquisition of Zn by roots and subsequently higher accumulation; 4) greater efficiency of Zn utilization at all levels of plant organization, including better distribution management and maintenance of the activity of enzymes normally dependent on Zn availability. So far, it is not clear whether these strategies are used by all plants, and if yes, what their relative contribution is in different species (Alloway, 2008). In wheat, the root system structure has been shown to have little effect on Zn efficiency, since vulnerable genotypes appeared to have better developed root systems and vice versa (unpublished PhD thesis of Holloway RE: "Zinc as a subsoil nutrient for cereals", University of Adelaide, 1996, as referred to by Alloway, 2008). Interestingly, another example from wheat suggests that elevated expression and activity of Zn-containing enzymes may be a primary mechanism of Zn efficiency in this species (Hacisalihoglu et al., 2003). This group has found no correlation of Zn efficiency with Zn uptake by roots, or with Zn transport from root to shoot. Other researchers have attached greater significance to the ability of plants to obtain Zn as compared to the strategy of reduced dependence on Zn for metabolic processes (Graham, 1984; Ruel and Bouis, 1998; Grotz and Guerinot, 2006). This implies a potentially immense role of root membrane transport proteins both for Zn uptake and for the exudation of phytometallophores. The correlation between Zn efficiency and release of Zn chelators has been well documented in wheat (Zhang et al., 1989; Cakmak et al., 1996) and rice (Hoffland et al., 2006), while no evidence for organic anion exudation with regard to Zn acquisition is available for the leguminous species. In rice, oxalate was the predominant phytometallophore extruded by roots. However, citrate, although less abundant, appeared to be more efficient in the mobilization of Zn (Hoffland et al., 2006). Recently, Xue et al. (2016) reviewed mechanisms of Zn and other nutrients acquisition in cereal/legume intercropping experiments. It appears that phytometallophores released by root systems of cereals, such as wheat, increase the bioavailability of soil Zn for chickpea and other legumes (Xue et al., 2016). In addition to the increased ability of plants to obtain Zn from the soil via excretion of chelating agents, maturation dynamics also seem to be an important factor determining Zn efficiency, at least in rice. Early-maturing rice genotypes tend to be less Zn efficient, because the developmentally conditioned high demand for Zn precedes the formation of

an adequate root system to meet that demand (IRRI, 1971; Giordano et al., 1974; Neue et al., 1998; Alloway, 2008). Nevertheless, in chickpea, early-flowering genotypes proved to be more Zn-efficient and vice versa (Khan et al., 2000). Siddiqui et al. (2013) concluded that plant growth (relative shoot dry matter) is not an appropriate parameter for determining the Zn efficiency of chickpea genotypes. Instead, Zn-accumulation capacity before flowering, which correlates very strongly with grain yield, may be a better estimator (Siddiqui et al., 2013). Earlier, Khan et al. (1998b) also emphasized the importance of elevated Zn-accumulation ability as a mechanism of Zn efficiency in chickpea, but pointed out that efficient root-to-shoot transport may also contribute to the efficiency (Khan et al., 1998b). Metallothioneins (MTs) are low-molecular-weight proteins thought to be implicated in Zn translocation and homeostasis in plants (Broadley et al. 2007). Recently, based on differential expression of MT-like genes after Zn application in coffee plants, Barbosa et al. (2017) suggested that MTs may play a role in the plant's adaptation to Zn-deficient conditions.

2.5.2. Genetic basis of Zn-dependent SNF efficiency
Despite recent revolutionary developments in the area of legume genomics, no attempt has been made to dissect molecular events underlying Zn efficiency with regard to SNF. It may appear to be mediated via general growth effects and accumulation of Zn pools sufficient for the maintenance of symbiosis. In other words, no "special" genes seem to exist for superior SNF under low Zn availability. However, some relevant examples from nonlegume species may challenge this assumption. Graham et al. (1992), Velu et al. (2017), and Yilmaz et al. (2017) provided evidence for independent genetic control of Zn efficiencies specific to various situations. In wheat, barley, and oat, Zn efficiency did not correlate with efficiencies for other micronutrients, such as Mn. Furthermore, the genetic basis for Zn efficiency on different soil types was also different. Finally, the genotypes that obtained Zn from nutrient-poor soils more efficiently also produced higher biomass and grain yield, but did not appear to be superior with regard to Zn content in leaves and seeds, indicating no genetic linkage between these traits (Graham et al., 1992). Recently, Velu et al. (2017) and Yilmaz et al. (2017) reconfirmed that Zn-deficiency tolerance in wheat is controlled independently from Zn content in grains. In order to confirm whether the effect on SNF is direct or mediated by other physiological processes, genes associated with higher SNF performance under conditions of low Zn supply need to be identified. It may be useful to know the approximate number and ontology of genes relevant to Zn transport and metabolism in a plant genome. Broadley et al. (2007) prepared a comprehensive inventory of Zn-related proteins in *Arabidopsis thaliana*.

It appears that 2367 proteins that belong to 181 gene families have features associated with Zn in this species. Proteins, according to their predicted molecular function, were distributed among various groups, namely binding (1503), catalytic activity (634), transcription regulator activity (379), transporter activity (254), molecular function unknown (241), signal transducer activity (26), structural molecule activity (12), translation regulator activity (10), and enzyme regulator activity (7). Can this information be extrapolated to chickpea proteins? Unlike chickpea, *A. thaliana* does not undergo endosymbioses such as association with arbuscular-mycorrhizal fungi and N-fixing symbiosis with rhizobia. Thus, the total number of protein-coding genes in chickpea is likely to be larger. The June 2016 release of *A. thaliana* genome annotation lists 27,655 protein-coding genes (https://www.arabidopsis.org), while the percent coverage of its genome by the initial release in 2000 was ca. 92% (Arabidopsis Genome Initiative, 2000). The recently updated genome annotation of desi-type chickpea contains 30,257 protein-coding genes, with 94% of estimated gene space captured by this sequencing effort (Parween et al., 2015). Thus, it is reasonable to expect an even larger number of Zn-related genes (>2367 proteins) in chickpea.

3. Chickpea biology and nodulation

3.1. General description of chickpea

Chickpea (*Cicer arietinum* L.) is an obligatory self-pollinating diploid annual herbaceous plant with $2n = 2x = 16$ chromosomes and a genome size of ca. 740 Mb (Gaur et al., 2010; Jain et al., 2013). It belongs to the Galegoid clade (cool-season or temperate clade) of the Papilionoideae legume subfamily, which also contains the model legume *M. truncatula* (Varshney et al., 2009). The crop originated from southeastern Turkey and Syria, with *Cicer reticulatum* Ladiz. being proposed as its wild progenitor (Kantar et al., 2007). It is cultivated mostly in arid and semiarid regions around the world, with temperatures between 5 and 25 °C and annual rainfall of 200–600 mm, on rain-fed soils (sandy to silt loam) with residual moisture (Rupela and Beck, 1990; Millan et al., 2006; Chibarabada et al., 2017). In the tropics, chickpea is grown in winter, while in temperate climates it is a summer or spring crop (Gaur et al., 2010). Although chickpea is generally considered to be a long-day plant (12 h or more; Chibarabada et al., 2017), it should be kept in mind that a photoperiod of 20 h inhibits nodulation in this crop (Dart et al., 1975; see also Section 3.3.3). Two major commercial groups of chickpea are recognized: kabuli-type and desi-type. Kabuli-type chickpeas have white flowers and large light-colored round seeds with a thin coat and smooth surface. Desi-chickpea varieties, with some exceptions, are generally characterized by pink flowers with anthocyanin

accumulation in the stem. The whole plant and leaves are smaller in size. Their angular seeds have a thick coat, vary considerably in color (shades of brown, yellow, green, and black), and are approximately half the size of kabuli seeds (Ahmad et al., 2005; Gaur et al., 2010). Chickpea cultivars have a large range of plant heights (20–100 cm). Some tall varieties can grow up to 130 cm under favorable conditions (Reddy et al., 1985; Singh, 1997). The plant has a deep and strong tap root system with a few lateral roots. The roots can penetrate some soil types up to 120 cm in depth (Sheldrake and Saxena, 1979; Singh, 1997). Chickpea seedlings emerge 7–15 days after sowing, depending on soil temperature and sowing depth. Their cotyledons remain underground (the hypogeal type of emergence). Vegetative growth before flowering generally ranges from 40 to 80 days and continues after flowering (the indeterminate growth habit). After fertilization, the pods are first visible in ca. 6 days. Within 10–15 days after the pod onset, intensive growth of the pod wall occurs, while seeds start growing later. For seed propagation, the harvesting should be conducted no earlier than the time point when ca. 90% of stems and pods turn light golden-yellow (Gaur et al., 2010). Chickpea plants can produce from a very few to over 1000 pods per plant (Pundir et al., 1992; Singh, 1997). The growth cycle of chickpea generally ranges from 84 to 125 days (Chibarabada et al., 2017).

3.2. Zn-deficiency symptoms and Zn requirement in chickpea

The first symptoms of Zn deficiency in chickpea under pot culture may become noticeable 3 to 4 weeks after planting. They include a reduction in plant height and a moderate chlorosis of leaves. Six weeks after planting, these initial symptoms worsen and are combined with a reduction in leaf size. Zn-deficient plants also have fewer branches. Leaflets of younger leaves acquire reddish brown pigmentation on their margins, which is followed by bronze coloration, necrosis, and premature abortion of leaflets and then the whole leaf. A characteristic feature of Zn deficiency in sensitive chickpea genotypes is the thickening of old leaves without apparent accumulation of water. Lack of Zn also causes delay in maturation in chickpea (Khan et al. 1998b; Kumar and Sharma, 2013). Kumar and Sharma (2013) also provided color plates illustrating different stages of Zn deficiency in chickpea. Shoot critical concentration of Zn associated with 90% of maximal growth was estimated between 20 and 21 mg kg^{-1} dry weight and did not appear to be different between a few genotypes contrasting for their Zn-use efficiency (Khan et al., 1998a). In that study, the shoot was reported to contain only 6.3 mg Zn kg^{-1} dry weight when the seed content and the experimental soil without fertilization were the sole sources of Zn for the plant. A lower critical value, namely 17 mg kg^{-1} dry weight in the youngest tissue, calculated based on 90% of

the relative yield, was reported by Brennan et al. (2001). Under nonsymbiotic conditions, between 0.48 and 0.70 mg Zn kg^{-1} soil (DTPA/pentetic acid-extractable Zn) appears to be sufficient for chickpea, while soils with more than 0.70 mg Zn kg^{-1} suppress chickpea yield (Singh and Gupta, 1986). Siddiqui et al. (2013) used 0.01 and 0.5 mg ZnSO$_4$ L^{-1} nutrient solutions to create Zn-deficient and Zn-sufficient conditions, respectively. Another study assessed sensitivity to Zn depletion in the soil with 0.06 mg kg^{-1} (DTPA-extractable Zn), while 2.5 mg Zn (ZnSO$_4$7H$_2$O) kg^{-1} soil served as a Zn-replete control (Khan et al., 1998b).

3.3. Chickpea nodulation

3.3.1. Description of nodulation parameters
Chickpea can obtain up to 80% of N for its growth from the air via symbiosis with rhizobia, soil bacteria that trigger the formation of specialized organs called root nodules. Under field conditions, nodules appear about 1 month after plant emergence. Their distribution is generally limited to the upper 15 cm of the soil. Under axenic culture conditions, nodules become visible at ca. 20 days after inoculation (Rupela and Dart, 1979). Unlike in some other legumes, such as pigeonpea, chickpea nodules are strongly attached to roots and therefore are more amenable to certain analyses (Rupela, 1990). Chickpea nodule morphology is of the indeterminate type, similar to the morphology of other Galegoid clade legumes, with clear developmental zones, such as found in well-characterized *M. truncatula* nodules (Kantar et al., 2007; Varshney et al., 2009). However, unlike *M. truncatula*, the chickpea N metabolism involves export of both amides and ureides from nodules (Thavarajah et al., 2005). Rhizobial infection in chickpea is thought to begin with the root-hair-type entry and continue with intercellular infection threads, from which rhizobia enclosed in a plant-derived membrane (symbiosomes) are released into the cytoplasm by an endocytosis-like mechanism. Infected cells of the N-fixation zone become densely packed with symbiosomes. Each symbiosome in chickpea typically contains a single differentiated rhizobial cell (bacteroid). Noninfected cells at the central area of chickpea nodules are smaller in size and highly vacuolated. A characteristic feature of chickpea nodule ultrastructure is the presence of electron-dense inclusions in the intercellular spaces of the N-fixation zone and also in plasmodesmata that connect infected and uninfected cells (Kantar et al., 2007). The shape of the nodules is initially elongated. Later, a permanently active apical nodule meristem may branch, forming a coral-like structure that can be up to 3 cm across (Dart et al., 1975). Individual nodules reach 3–4 mm in length (Aouani et al., 2001). The number of nodules per plant may lie within the following ranges, depending on chickpea genotype, rhizobial strain, and growth conditions: 2–14 (Gul et al., 2014), 8–38 (Khurana

and Dudeja, 1996), 13–30 (Ben Romdhane et al., 2007), 20–36 (Aouani et al., 2001), or 21–101 (Biabani et al., 2011). Nodule dry weight per plant varies between 60 and 500 mg (Aouani et al., 2001). There is a report of a twofold difference in root length density between high-nodulating (32 m per plant) and low-nodulating (ca. 16 m per plant) chickpea genotypes in a pot trial (Rupela, 1994).

3.3.2. Dynamics of nodule activity and correlation between growth parameters
Leghemoglobin red coloration becomes visible in 2-week-old chickpea nodules (Aouani et al., 2001). Senescence of nodules starts at the nodule base with the formation of a brown or green zone, which broadens with further nodule growth. The longevity of nodules in chickpea depends much on environmental conditions. In one field study in India (Rupela and Dart, 1979), nodule N-fixing activity (acetylene reduction assay, ARA) was lost by 89 days after planting at a location close to Hyderabad (south-central India), whereas at Hisar (north India) nodule activity persisted up to 145 days after planting, which corresponds to about 3 weeks prior to the final seed harvest at both locations. This study provides further details on the dynamics of nodule activity among five chickpea cultivars. Whereas the nitrogenase activity (ARA) per plant per hour, as well as nodule number and weight, was the highest by 61 days after planting, the specific nitrogenase activity (per gram dry weight nodule per hour) was the greatest in young nodules, namely 17 days after planting (Rupela and Dart, 1979). In addition, the authors recorded a strong correlation between nitrogenase activity (per plant per hour) and nodule number and weight (Pearson correlation coefficients 0.778 and 0.763, respectively). Similar correlation values were reported for nitrogenase activity (per plant per hour) and nodule number and weight (0.650 and 0.840, respectively) in a study by Rupela (1990). However, it would be inaccurate to substitute the nitrogenase activity measurements by these two easily scorable parameters, since the opposite relationship was reported in the symbiosis between Syrian chickpea variety ILC1919 and the *Mesorhizobium ciceri* ch-191 strain (Tejera et al., 2006; Kantar et al., 2007). It should be noted that, unlike the nitrogenase activity expressed per plant per hour, the specific nitrogenase activity (per gram dry weight nodule per hour) did not appear to correlate well with nodule number and weight (Rupela, 1990). Likewise, N-fixation rates in chickpea assessed by the percentage of total N and the $^{15}N/^{14}N$ isotope ratio method correlated very weakly with nodule number and weight (Biabani et al., 2011). Biabani et al. (2011) also emphasized that nodule number taken alone is a poor estimator of SNF effectiveness in chickpea and advocated the simultaneous use of several independent characteristics. Only a weak to moderate correlation was found between shoot

weight and nitrogenase activity, as measured by the ARA (Rupela, 1990). Interestingly, a very weak to weak negative correlation was observed between the chickpea shoot N content and the nodule size and number in a study by Qureshi et al. (2013).

3.3.3. Environmental factors, high N levels, and nutrient deficiencies influence nodulation in chickpea

Low availability of water, suboptimal temperatures, long days, excessive salinity, and high amounts of N in the soil greatly affect nodulation in chickpea (Dart et al., 1975; Rupela and Saxena, 1987; Elsheikh and Wood, 1990; Ben Romdhane et al., 2009). On the other hand, SNF in chickpea is negatively influenced by the deficiency of such nutrients as P, Fe, Mo, Co, B, and Zn (Yadav and Shukla, 1983; Yanni, 1992; Khan et al., 2014; Esfahani et al., 2016). Both the nodule number and the diversity of chickpea-nodulating rhizobia were adversely influenced by drought in a study by Ben Romdhane et al. (2009). In another study, however, nodule dry weight, but not nodule number, decreased after exposure to water-deficient conditions (Esfahani and Mostajeran, 2011). Soil temperatures below 15 °C and above 25 °C are thought to be detrimental for SNF in chickpea (Rupela and Beck, 1990). Dart et al. (1975) reported temperatures close to 23 °C as being optimal for chickpea nodule development and N fixation, while the nitrogenase activity (ARA) in their experiments was maximal between 24 and 33 °C, with a steep decline at higher temperatures. Nodule formation was completely abolished at 33 °C. The researchers concluded that the inhibition of nodule functioning at temperatures above 30 °C was due to a decrease in the amount of nitrogenase enzyme present and possibly related to higher rates of basal nodule senescence, but not due to the absence of rhizobia. Dart et al. (1975) also examined the effect of the photoperiod on nodulation in chickpea. They reported an adverse influence of a 20-h light regime on nodulation as compared to 11-h day length, which was attributed to general plant vigor and accelerated senescence of the nodule base rather than to the decrease of nitrogenase activity (Dart et al., 1975). Salinity reduces the nodule number and weight in chickpea already at low levels of salt (1.0 dS m^{-1}, equivalent of 8.6 mol m^{-3} NaCl), while 7 dS m^{-1} (equivalent of 63.3 mol m^{-3} NaCl) completely inhibited nodule formation (Elsheikh and Wood, 1990). An effect of soil N on nodulation in chickpea was reported by several groups. A 50% reduction in nodule number was observed in a pot culture experiment by Rawsthorne et al. (1985) when plants were supplied with 1.43 mM NO$_3^-$. Higher tolerance, however, was observed by Jessop et al. (1984), who examined the nodulation characteristics of chickpea at five levels of soil NO$_3^-$ in a controlled-environment experiment. They found that 3.0 mM NO$_3^-$

was optimal for nodule development and N fixation, while too little (0 mM and 0.75 mM) and too much (6 mM) NO$_3^-$ was associated with lower nodule mass and lower nitrogenase activity (ARA, per plant per hour) early in the development (56 days after sowing). At the same time, 6 mM NO$_3^-$ improved the nodule number later in the development (90 days after sowing). The authors pointed out that chickpea nodulation appears to be less sensitive to high N levels than nodulation in soybean (Jessop et al., 1984). The negative effect of excessive N on nodulation in chickpea was also observed in field studies. Sheoran et al. (1997) reported that application of 100 kg N per hectare results in reduced nodule biomass compared to no extra N added. At the same time, the elevated N level significantly improved total plant N and grain yield (by 8.6%–28.4%) in this study, which emphasizes a dilemma of choice between nonsustainable higher profits and low-input cropping designs that take into account long-term effects on the soil and water ecosystems. Another study, however, showed that an even greater increase in grain yield can be achieved due to inoculation of chickpea with rhizobia (70%–72%), which is comparable with the benefits from N application at a rate of 50 kg N per hectare (El Hadi and Elsheikh, 1999). Rupela and Beck (1990) reported a 4–6-fold reduction in nodule weight with an increase of NO$_3^-$ concentration in the top 15-cm soil layer from 6 mg kg^{-1} soil to 13 mg kg^{-1} soil. Similar results, although with lower magnitude of reduction, were reported by Rupela (1994). Their field study suggested that 10 mg total N kg^{-1} soil (or less) may be the best for nodulation performance in chickpea. Finally, as briefly mentioned in Section 2.3, nodulation in chickpea requires adequate amounts of available Zn. Several studies provided different figures for the optimal amount of Zn for chickpea nodulation. One field study reported the optimal Zn application dose to be 25 kg ZnSO$_4$ ha^{-1} (Das et al., 2012). Singh et al. (2014) recommend using 20 kg of Zn ha^{-1} in combination with rhizobia as a treatment optimal for both nodulation and yield characteristics. Two other studies provided the SNF-optimal Zn concentration in a form more relevant for controlled-environment experiments. Yadav and Shukla (1983) reported a critical range of Zn for chickpea nodulation within 1.75–14.0 mg kg^{-1} soil, with the optimum between 5 and 10 mg kg^{-1} soil, where Zn amount indicates DTPA-extractable Zn. Another study demonstrated that as much as 20 mg Zn kg^{-1} soil ensures good nodulation in chickpea (Misra et al., 2002).

3.4. Chickpea genotypes potentially useful in screening for Zn-dependent SNF efficiency

Superior SNF under Zn-depleted conditions may or may not be related to the efficiency of Zn use alone or the degree of symbiotic performance at normal Zn levels. In

any case, screening for the combination of these two traits may be conducted among genotypes previously tested for these characters. In addition, such genotypes may serve as controls for the analysis of chickpea germplasm with unknown efficiencies. Table 1 lists chickpea genotypes characterized for their tolerance to Zn starvation. Table 2 contains information on chickpea lines with known SNF properties. As mentioned in Section 1, at least some high-nodulating chickpea genotypes tend to be more susceptible to fungal diseases. This circumstance not only decreases the potential value of such genetic material for cropping but also imposes substantial difficulties at the screening stage, which is typically conducted in a greenhouse or a growth chamber, environments that are highly prone to fungal outbreaks. Thus, screening for a symbiotically efficient germplasm should be conducted among genotypes resistant to fungal pathogens (Khurana and Dudeja, 1996). We have listed a subset of such genotypes in Table 3. Although most of these lines confer resistance to *Ascochyta* blight, genetic makeups unsusceptible to other fungal pathogens were also identified in high numbers, which should be sufficient for medium-scale screening.

3.5. Rhizobial strains compatible with chickpea

3.5.1. Current taxonomic status of chickpea-nodulating rhizobia

Chickpea was traditionally considered a very selective host for nodulation, primarily because it cannot interact with highly promiscuous rhizobia, such as *Rhizobium* sp. NGR 234 (Broughton and Perret, 1999; Perret et al., 2000). *Mesorhizobium ciceri*, *M. mediterraneum* (Nour et al., 1995), and *M. muleiense* (Zhang et al., 2014) were described as specific microsymbionts of chickpea. Later it was found that the range and genetic diversity of rhizobial species capable of forming symbiosis with chickpea are less limited. Rhizobial strains isolated from nodules of chickpea grown in various climatic zones were related to the following species: *M. loti* (Maatallah et al., 2002; Laranjo et al., 2004), *M. amorphae* (Laranjo et al., 2004; Alexandre et al., 2009), *M. tianshanense* (Alexandre et al., 2006; Rivas et al., 2006), *M. temperatum* (Brigido et al., 2007; Dudeja and Singh, 2008), *M. huakuii* (Alexandre et al., 2009), and two promiscuous nodulators, *Ensifer medicae* (formerly *Sinorhizobium medicae*) and *E. meliloti* (formerly *S. meliloti*), which are not effective in N fixation

Table 2. Chickpea genotypes tested for their nodulation characteristics.

#	Chickpea genotypes				References
	High-nodulating	Moderate-nodulating	Low-nodulating	Nonnodulating	
1	K 850	-	-	-	Rupela, 1990
2	K 850 and H 75-35	BG 209, Pant G 114, and C 235	L 550 and H 208	-	Khurana et al., 1991
3	High-nodulating and low-nodulating plants were identified from four cultivars: ICC 4948, ICC 5003, ICC 14196, and Kourinski			ICC 435, ICC 4918, ICC 4948, ICC 4993, and ICC 5003	Rupela, 1994
4	ICC 4948HN and ICC 5003HN	-	ICC 4948LN and ICC 5003LN	-	Khurana and Dudeja, 1996
5	CP92296 (parent ICCV 91019), CP92252 (parent ICCV 91016), and other selections from ICCV 91019, ICCV 91016, ICCV 91026, and from ICC 4958	-	-	-	Rupela, 1997
6	MCA103†, MCA131, and MCA250	MCA31 and MCA45	MCA301, MCA370, Rizky, and Douyet	-	Sadiki and Rabih, 2001
7	Sirio and Gulavi	Pedrosillano and ILC1919#	-	-	Tejera et al., 2006
8	ICC 4948HN and ICC 5003HN	-	ICC 4948LN and ICC 5003LN	ICCV 2NN, ICC 435NN, ICC 4918NN, and ICC 4993NN	Upadhyaya et al., 2006
9	254549 = ILC 235 [0.084], 451161 = RPIP 12-071-03831 [0.060], and 339223 = ILC 263 [0.059]§	See details in the reference	451420 = RPIP 12-071-04815 [0.006], 360439 = ICC 6990 [0.020], and 359429 = ICC 6618 [0.021]§	-	Biabani et al., 2011
10	-	-	-	ICC 19181, ICC 19183, ICC 4993, and ICC 4918	Gul et al., 2011

† This and other genotypes selected in this study were ranked for SNF performance under salt stress, taking into account yield-related traits.
# Genotype ILC1919 was the most tolerant to salt stress with regard to SNF in this study.
§ Only the top three and the bottom three genotypes are listed here (out of 40), based on total N fixed (indicated in square brackets).

Table 3. Some chickpea genotypes with resistance to fungal pathogens.

#	Resistant/moderately resistant chickpea genotype	Fungal pathogen (resistance)	References
1	ICC# 202, 391, 658, 858, 1443, 1450, 1611, 3439, 4552, 6098, 6671, 8933, 10130, 11088	*Fusarium* wilt	Nene and Haware, 1980
2	AKN33, AKN42, AKN98, AKN99, AKN102, AKN144, AKN145, AKN146, AKN147, AKN148, AKN395, AKN411, AKN426, AKN568, 87AK71114, ESER87, İZMİR92, MENEMEN, DAMLA89, GÖKÇE, KÜSMEN99, ER99, UZUNLU99, AKÇİN91, SARI98, AYDIN92, AZİZİYE94, FLIP 84-92C(3)	*Ascochyta* blight	Cingilli et al., 2003
3	ILC3279	*Ascochyta* blight	Millan et al., 2003
4	Hashem and ILC-482£	*Ascochyta* blight	Younesi et al., 2004
5	Resistant both at seedling and at pod formation stages: 92A048, NB 02169, NB 02173, NB 02175, NB 02178, NB 02179, NB 02180, NB 02181, NB 02183, NB 02184, ILC-7374, FLIP97-132C, FLIP98-176C, FLIP98-226C, FLIP99-54C, FLIP00-50C, FLIP00-55C, KR-4, FLIP98-198C, FLIP98-80C, FLIP97-195C, X98TH10, SEL96TH11507, NCS-9905, NC9903, NC9904, Dasht, Parbat, Balkasar, NIFA-88	*Ascochyta* blight	Iqbal and Ghafoor, 2005
6	Lines released in different countries, with acceptable degree of resistance: ILC 72 (Califfo and Fardan), ILC 195 (Giza 195), ILC 200 (Zegri), ILC 202, ILC 237, ILC 411, ILC 464 (Kyrenia), ILC 482 (TS 1009, Rafidain, Jubeiha 2, Janta 2, Ghab 1, Güney Sarısı 482), ILC 484, ILC 533 (Elixir), ILC 915 (Jebel Marra - 1), ILC 1335 (Shendi), ILC 2548 (Almena), ILC 2555 (Alcazaba), ILC 3279 (Yialosa, Dijla, Sultano, Jubeiha 3, Ghab 2, Chetoui), ILC 6188 (Ali)¥; resistant to six races of *Ascochyta*: ILC 4475, ILC 6328, ILC 6482, ILC 12004; further 68 resistant genotypes listed and 1584 resistant genotypes referenced	*Ascochyta* blight	Pande et al., 2005
7	Moderately resistant: ICC# 1915, 6306, and 11284	*Ascochyta* blight	Pande et al., 2006
	Moderately resistant: ICC# 1180, 2990, 4533, 4841, 4872, 6263, 6279, 6877, 7255, 7323, 7308, 7315, 7554, 7571, 7668, 7819, 8151, 8261, 8318, 8740, 8855, 9137, 9402, 9848, 9862, 10341, 10755, 10885, 11284, 11764, 11879, 12028, 12037, 12155, 12328, 12492, 13124, 13187, 13219, 13283, 13357, 13461, 13599, 13628, 13816, 14199, 14595, 15264, 15294, 15333, 15406, 15435, 15697, 15802, and 16796	*Botrytis* gray mold	
	Moderately resistant: ICC# 1710, 2242, 2277, 11764, 12328, and 13441	Dry root rot	
	Immune: ICC# 637, 1205, 1356, 1392, 2065, 2072, 2629, 2990, 3218, 4495, 4533, 5639, 6279, 7184, 8058, 13219, 14402, 14669, 16207, 16374, and 16903; resistant: ICC# 67, 95, 791, 867, 1164,1398, 2210, 3230, 6571, 6811, 6816, 6874, 7554, 7819, 9848, 11584, 11664, 12028, 12155, 13441, 13599, 13816, 14815, 14831, and 15868; moderately resistant: ICC# 1397, 1431, 1510, 1715, 1923, 3325, 4593, 5135, 5845, 7867, 8950, 9002, 10393, 12307, 12916, 12928, 12947, 15567, 15606, 15610, and 16487	*Fusarium* wilt	
8	ICC# 184, 229, 338, 342, 1246, 1405, 2104, 2595, 4928, 5535, 5901, 11223, 11224, 11312, 11318, 11321, 11322, 11324, 11550, 11554, 12233, 12235, 12237, 12242, 12246, 12248, 12251, 12253, 12258, 12259, 12267, 12268, 12270, 12273, 12289, 12428, 12430, 12431, 12435, 12440, 12450, 12452, 12454, 12472, 14344, 14364, 14366, 14368, 14369, 14371	*Fusarium* wilt	Upadhyaya et al., 2006
	ICC# 11088, 11315, 12269, 12437, 14440, 14449	Dry root rot	
	ICC# 12274, 12275, 14411, 14425, 14426, 14444, 14450, 14451	Black root rot	
	ICC# 344, 542, 618, 684, 1696, 4709, 9934, 14282, 14391	Collar rot	
	ICC# 1084, 1102, 3540, 4018, 4065, 4075, 6671, 12512	*Botrytis* grey mold	
	ICC# 652, 1929, 3864, 4063, 12955, 12965, 14912, 14915, 14917, 15973, 15975, 15978, 17000	*Ascochyta* blight	
	ICC# 403, 685, 693, 1136, 2546, 3718, 6433, 10495	Stunt	
9	Resistant: 101, 620; moderately resistant: 08-AG-004, CH-70/02, CH-76/02, NOOR-91, Paidar-91, Pb-2000, 818, 870	*Ascochyta* blight	Ali et al., 2013
10	Resistant: PBG 5, H08-93, GLK 26167, and JGK 13(R); moderately resistant: Phule G 09103, GNG 1888, CSJK 6(R), Phule G 09316, Kripa (Phule G 0517), and BG 3012(R)	*Alternaria* blight	Manjunatha and Saifulla, 2013
11	Resistant: 8032, Thal-2006, 06001, and 5CC-109; moderately resistant: Bital-98, 03008, PB-2000, and Noor-91	*Ascochyta* blight	Rashid et al., 2014

£ The two genotypes were tolerant to some of the 30 isolates tested in this study.
¥ Commercial names are shown in brackets.

(Aouani et al., 2001; Ben Romdhane et al., 2007, 2009). Chickpea can also be effectively nodulated by *Rhizobium leguminosarum* strains (Kantar et al., 2003; Gul et al., 2014). The ability to interact with these distantly related rhizobia may be due to the high similarity of symbiotic genes *nodC* and *nifH*, which are shared by chickpea rhizobia via lateral gene transfer (Laranjo et al., 2008). Sequences of *nodC* (Nod-factor production) and *nifH* (nitrogenase) genes in at least five *Mesorhizobium* species, namely *M. ciceri*, *M. mediterraneum*, *M. loti*, *M. amorphae*, and *M. tianshanense*, are virtually the same, which may be associated with production of similar Nod-factors specifically recognized by chickpea (Laranjo et al., 2008; Alexandre et al., 2009).

3.5.2. Tolerance of rhizobia to environmental stresses and Zn toxicity

Chickpea rhizobia are sensitive to a number of environmental factors, such as heat, low soil pH, water deficiency, salinity, heavy metals, soil nitrate, and biocides, including commonly used greenhouse fungicides (Bottomley, 1991; Walsh, 1995; Kyei-Boahen et al., 2001; Kantar et al., 2007). Although optimal growth of most chickpea rhizobia occurs at 28 °C, some strains prefer 20 °C. Lower temperatures (20 °C) are better tolerated than higher temperatures (37 °C). The maximal temperature range for their growth is 30–40 °C. Unfortunately, chickpea rhizobia more tolerant to suboptimal temperatures exhibit lower symbiotic efficiency (Maatallah et al., 2002; Rodrigues et al., 2006). The choice of an optimal Zn concentration for SNF screening under Zn-replete conditions (control) must also take into account the sensitivity of rhizobia to Zn toxicity, as discussed in Section 2.4. In liquid culture, only a few chickpea-specific strains can tolerate $ZnCl_2$ at a concentration of 50 µg mL^{-1} (Maatallah et al., 2002). For rhizobia in their free-living form (field), the lowest observed effect concentrations of Zn ranged from 90 to 876 mg kg^{-1} soil among 11 dedicated studies on *R. leguminosarum* (Broos et al., 2005). This concern, however, may be of little relevance for experiments under pot culture conditions, even if the metal gets accumulated in pots over the growth period. Typical Zn content in plant nutrient solutions used in representative chickpea nodulation studies lies within the range of 0.08–10 µM $ZnSO_4$, which corresponds to only 0.013–1.6 µg $ZnSO_4$ mL^{-1} (Balasubramanian and Sinha, 1976; Jessop et al., 1984; Maatallah et al., 2002; Tejera et al., 2006; Biabani et al., 2011). One important feature of chickpea rhizobia to be aware of while setting up a screening assay is their genetic instability during storage on agar-based media. This instability can result in the loss or modification of their original symbiotic properties (Thies et al., 2001; Naseem et al., 2005). Therefore, special attention should be paid to the reliability of the strain supplier, adequate shipment, maintenance at –70 °C, and

rigorous monitoring of phenotypic properties of newly acquired rhizobial strains (Kantar et al., 2007).

3.5.3. Choice of chickpea-specific strains suitable for a screening experiment

Under field conditions, inoculation with rhizobial cultures has been shown to result in a better grain yield and N content, the magnitude of this benefit being comparable with the application of nitrogenous fertilizers (Sheoran et al., 1997; El Hadi and Elsheikh, 1999; Ben Romdhane et al., 2008). The efficiency of such inoculation depends very much on matching chickpea genotypes to proper rhizobial strains (Kantar et al., 2007). Substantial variation in symbiotic properties is present not only among chickpea lines, but also in chickpea-specific rhizobial isolates (Maatallah et al., 2002; Ben Romdhane et al., 2007; Biabani et al., 2011). The effectiveness of applied rhizobia may be quite dissimilar in different environments and is influenced by their ability to compete with rhizobia already present in a particular field (Sheoran et al., 1997; Ben Romdhane et al., 2007). In fact, superior SNF efficiency of a strain does not necessarily correlate with its competitiveness (Amarger, 1981). Therefore, the optimal combination of these parameters must be found on a case-by-case basis, by careful selection among strains indigenous to the prospective production area (Kantar et al., 2007; Ben Romdhane et al., 2008). For experiments in a controlled environment, such as advocated in this review, a different set of criteria must be used to meet the goals of a study. Namely, medium- to large-scale screening for Zn-dependent SNF characteristics in a population of genetically distant chickpea lines requires inoculation with a rhizobial strain, or a mixture of strains, which guarantees a relatively uniform degree of interaction with the host. Biabani et al. (2011) used mixed inoculation in order to assess nodulation potential in a subset of 40 chickpea genotypes representative of the global chickpea germplasm collection. In this pot culture study, an equal colony-forming unit mixture of *M. ciceri* strains USDA3378, USDA3379, and USDA3383 was applied as an inoculum. Gul et al. (2014) characterized 47 chickpea genotypes collected worldwide for their nodulation and seed yield under pot culture conditions. Their work was based on a commercial inoculum containing *R. leguminosarum*. Unfortunately, the authors provided no further detail as to the identity of the strain(s). The largest number of chickpea genotypes (155), most of which, however, came from a single geographic region (Ethiopia), were screened under field conditions by Keneni et al. (2012) using one symbiotically efficient strain of *Rhizobium* sp. CP EAL 004. Two other studies conducted field screening for high, medium, low, and nonnodulating variants among a few previously selected chickpea genotypes at different N levels. One of them applied *Rhizobium* sp. strain IC59

at sowing (Rupela, 1994), while the other used *Rhizobium* sp. strain Ca181 for coating seeds (Khurana and Dudeja, 1996). Many other strains, predominantly of *M. ciceri* background, were used for inoculation of individual chickpea genotypes at various conditions. Table 4 contains a list of strains that were used in combination with specific chickpea varieties since 1990. Some of these strains were reported as superior nodulators. However, their use for screening of genetically diverse chickpea germplasm may require preliminary testing.

Table 4. A list of representative nodulation studies on chickpea since 1990.

#	Rhizobial strains	Chickpea genotypes	Purposes of use/explanations	References
1	*M. ciceri* Ch191	ILC 482	Tolerance to salinity. Ch191 is a highly efficient salt-tolerant strain.	Elsheikh and Wood, 1990
2	*Rhizobium* sp. CM-1	H 75-35, BG 209, H 208, Pant G 114, K 850, C 235	Nodule occupancy under different conditions.	Khurana et al., 1991
3	*Rhizobium* sp. TAL 1148, TAL 480, TAL 620	Baladi, Gabel marra, NEC 25–27, NEC 2010, ILC 1919, Flip 85–108	Inoculation and N fertilization effect on yield and protein content. TAL 1148 was the most efficient out of the three strains.	El Hadi and Elsheikh, 1999
4	*M. ciceri* UPMCa7^T and *M. mediterraneum* 918	Amdoun I	Reference strains in effectiveness tests.	Aouani et al., 2001
5	*Mesorhizobium ciceri* CP 39	Myles	Effect of fungicides on survival of rhizobia.	Kyei-Boahen et al., 2001
6	*Rhizobium* sp. Ca181 and CH9160	ICC4948 and ICC5003	Control strains for high-nodulating and low-nodulating selections of chickpea.	Chaudhary et al., 2002
7	*M. ciceri* CP 39, 27A2, 27A7, 27A9 (commercial inoculum)	Myles and Sanford	Comparison of inoculation methods.	Kyei-Boahen et al., 2002
8	*R. leguminosarum* subsp. *ciceri* HF 274 and HF 177	Aziziye-94	Inoculation effect on yield. HF 274 and HF 177 are highly efficient strains, even in cold highland areas (Turkey).	Kantar et al., 2003
9	*M. ciceri* Ch191	Pedrosillano, Sirio, Gulavi, Lechoso, ILC1919	Tolerance to salinity. Ch191 is a highly efficient salt-tolerant strain.	Tejera et al., 2006
10	*M. ciceri* UPMCa7^T and CMG6, *M. mediterraneum* UPMCa36^T	Amdoun I, Kasseb, Chetoui	Test strains (UPMCa7^T and CMG6) and positive control (UPMCa36^T) in effectiveness tests. UPMCa7^T was not competitive in field trials (Tunisia).	Ben Romdhane et al., 2007
11	*M. ciceri* C-2/2	ILC-482	Effect of coinoculation with *Pseudomonas jessenii* PS06 (a phosphate-solubilizing bacterium) on growth and seed yield.	Valverde et al., 2006
12	*M. mediterraneum* CTM226 and *M. ciceri* CMG6	Amdoun I, Beja, Kasseb, Chetoui	Two strains with high symbiotic performance and salt tolerance.	Ben Romdhane et al., 2008
13	*Rhizobium* sp. Ca-220	BARI Cho1a-3, BARI Cho1a-4, BARI Cho1a-5, BARI Cho1a-6	Inoculation effect on nodulation and yield on calcareous soils. Genotype BARI Cho1a-3 showed superior nodulation characteristics.	Bhuiyan et al., 2008
14	*M. mediterraneum* LN707b and LN7007, *Rhizobium* sp. Ca181 and IC76	ICC 4948 and ICC 5003	Effectiveness tests on high-nodulating and low-nodulating selections of chickpea. Ca181 and IC76 were used as a reference. LN707b and LN7007 were the most efficient among isolated strains.	Dudeja and Singh, 2008
15	*M. mediterraneum* LILM10, *M. ciceri* CMG6	Amdoun I and Chetoui	Test strain (LILM10) and positive control (CMG6) in effectiveness tests under high salinity. LILM10 is a highly efficient salt-tolerant strain.	Ben Romdhane et al., 2009
16	*M. ciceri* C-15 and CP-36	Bivanij	Drought tolerance. Local strain (C-15) was more drought-tolerant and efficient than nonlocal strain (CP-36).	Esfahani and Mostajeran, 2011
17	*M. mediterraneum* LN-7007	Pant G-186	Analysis of inoculation and micronutrients (Zn, B, Mo) application effects on growth and yield.	Das et al., 2012
18	*Mesorhizobium ciceri* LMS-1 (pRKACC), transgenic	ELMO and CHK3226	LMS-1 (pRKACC), a transgenic ACC deaminase expressor (*acdS* from *Ps. putida* UW4), was associated with better symbiotic performance and lower susceptibility to fungal infection.	Nascimento et al., 2012
19	*M. ciceri* C-15, C-22, IC-59, CP-36, Ch-191, SWRI4, SWRI7, *M. mediterraneum* SWRI9	Bivanij	Comparative efficiency under N-limited conditions. C-15 exhibited superior performance, while Ch-191 and CP-36 were the least efficient.	Esfahani et al., 2014
20	*M. muleiense* CCBAU 83963^T, *M. ciceri* USDA 3378^T, *M. mediterraneum* USDA 3392^T	Kabuli	*M. mediterraneum* and *M. ciceri* were more competitive in sterilized substrates. *M. muleiense* was the predominant nodule occupier in soils native to the site of isolation (China).	Zhang et al., 2014
21	*M. ciceri* ENRRI8, USDA3100, and TAL 620	Salwa and Burgeig	Comparative efficiency. The three strains had comparable performance.	Mohamed and Hassan, 2015
22	*M. ciceri* CP-31, *M. mediterraneum* SWRI9	Bivanij	Comparative efficiency under P-limited conditions. CP-31 was more efficient.	Esfahani et al., 2016
23	*M. mediterraneum* UPM-Ca36^T, transgenic	ELIXIR (cultivar CHK 3236)	Transgenic strain overexpressing the native *clpB* chaperone gene was superior in nodulation characteristics.	Paço et al., 2016

4. Proposed experimental setup

An initial screening should be aimed at identification of a few chickpea genotypes with maximal differences in their SNF performance under Zn-limited conditions. The plants may be inoculated with a mixture of *M. ciceri* strains USDA3378, USDA3379, and USDA3383, as was done in the study by Biabani et al. (2011). This phase can include all or a portion of the genotypes listed in Tables 1, 2, and 3. Five to 10 plants can represent each genotype. The whole plant set should be supplied with a Zn-deplete nutrient solution to ensure soil Zn concentrations slightly above 1.75 mg Zn kg^{-1} soil, which is the minimum required for SNF in chickpea (Yadav and Shukla, 1983). Primary assessment of the symbiotic performance may be based on overall plant growth. Genotypes showing marked differences in growth as a group (with little variation between individual plants) may be selected for growth in Zn-replete conditions in order to ensure that growth differences are related to Zn nutrition. The Zn-sufficient environment should contain ca. 10 mg Zn kg^{-1} soil, which is the upper optimum limit for SNF in chickpea (Yadav and Shukla, 1983). At this stage, Zn-inefficient plants should largely recover their growth, while Zn-efficient plants should show the same or better development compared to their performance in Zn-deplete conditions. Genotypes that remain stunted at normal Zn supply levels should not be considered further. At the next stage, preselected genotypes can be grown in a larger number (e.g., 30 plants per genotype) for measurements of the nitrogenase activity via ARA (Tejera et al., 2006), qRT-PCR for *nifD*, *nifK* (Esfahani et al., 2016), or other reliable SNF marker genes in nodules. Measurements of the nodule dry weight and number on a subset of plants should also be conducted, as these parameters were found

to correlate with N-fixation rates measured via ARA in several studies (see Section 3.3.2.). Genotypes that exhibit extremes in sensitivity of SNF-related parameters to Zn deficiency, in parallel with the overall growth differences, may be selected for the RNAseq sample preparation. This phase should include four groups of plants: two of the most sensitive and two of the most tolerant genotypes grown in parallel in Zn-deplete and Zn-replete conditions. Nodules from each group should be collected in three biological replicates for RNA collection. We recommend using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) to ensure RNA extraction quality sufficient for RNAseq application. Thus, the overall sample number will be 24: two Zn levels for each of the four genotypes multiplied by three biological replicates. This sample number is suitable for a single run of a next-generation sequencing machine, such as NextSeq 500 (Illumina), and may cost about $16,000 (as of 2016) for external users at research institutions, such as the Noble Research Institute, OK, USA (Dr Yuhong Tang, personal communication). After assembly of the RNAseq data with the Trinity software (Grabherr et al., 2011; Haas et al., 2013), genes differentially expressed under Zn-deplete and Zn-replete conditions but showing comparable expression at normal Zn levels can be selected for further analysis. A short overview of the proposed experimental setup is presented in Table 5.

In summary, SNF in chickpea depends on Zn availability and possibly on the optimal use of this micronutrient. Exact mechanisms of Zn-use efficiency with regard to SNF in chickpea and other legumes remain unknown. Tolerance of various plant traits to Zn deficiency has a distinctive genetic basis, which is individual for different traits. It is conceivable that the

Table 5. Main stages of the proposed experimental setup.

#	Step	Description
1	Primary screening	Selection of SNF-efficient and SNF-inefficient genotypes under Zn-deplete conditions (1.75 mg Zn kg^{-1} soil). Assessment of the overall plant growth. 5–10 plants per genotype.
2	Secondary screening	Confirmation of SNF-efficient and SNF-inefficient genotypes under Zn-replete conditions (10 mg Zn kg^{-1} soil). Assessment of the overall plant growth. Elimination of genotypes with stunted growth. 5–10 plants per genotype.
3	Advanced screening	Detailed characterization of SNF parameters for preselected genotypes. Nitrogenase activity measurements (ARA), qRT-PCR (*nifD*, *nifK*), nodule number, and dry weight. 30 plants per genotype.
4	Growth for RNA extraction from nodules	Two very sensitive and two very tolerant genotypes should be grown under Zn-deplete and Zn-replete conditions in a large number (three biological replicates per variant) in order to generate 24 RNA samples suitable for a single run of a next generation sequencer (e.g., NextSeq 500, Illumina).
5	RNA preparation for RNAseq	Isolation of total RNA from mature N-fixing nodules (ca. 61 day after planting) with the Spectrum Plant Total RNA Kit (Sigma-Aldrich).
6	Sequencing of total RNA (RNAseq)	A single sequencing run of 24 samples with, e.g., NextSeq 500 (Illumina).
7	Assembly of transcripts	Following the sequencing quality assessment and data filtering, clean reads can be assembled with the Trinity software (Grabherr et al., 2013; Haas et al., 2013).
8	Bioinformatic analysis	Identification of differentially expressed transcripts (genes differentially expressed under Zn-deplete and Zn-replete conditions, but showing comparable expression at normal Zn levels).

SNF dependence on Zn is also controlled by a dedicated genetic program. To understand this regulation, genotypes strongly contrasting for Zn-related SNF performance must be identified in the course of special screening. Knowledge of chickpea biology, nodulation characteristics, and symbiotic partners is vital for such an undertaking. Therefore, these topics were addressed in detail in this review. Zn biology and Zn efficiency mechanisms were discussed in the context of their potential role in SNF. This discussion is relevant for the interpretation of subsequent transcriptomic studies that should follow the identification of suitable genotypes. Less attention was given to growing conditions for chickpea and to details on cultivation of chickpea rhizobia. Such information, however, is crucial for setting up an adequate experimental environment and deserves a separate comprehensive summary. Literature sources referenced in Section 3 can be used as a basis for such a review. Experimental procedures described for similar screening efforts in some of the listed studies can be adopted with minor modifications.

Acknowledgments

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