IDENTIFICATION OF ZINC BINDING PROTEINS OF WHEAT SEED

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IDENTIFICATION OF ZINC BINDING PROTEINS OF WHEAT SEED

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Abstract

Zinc content of the wheat seed has significance for agronomy, environment and also for human health. Seeds containing higher amounts of micronutrients, especially zinc, are better at germination and show high resistance to pests. Experiments on the mechanisms of micronutrient uptake and their accumulation in the plant body and the seeds showed a correlation between zinc and protein amount in the seed, which suggested some proteins, mostly abundant in the embryo and the aleurone layer, might be sinks for zinc. There is a lack of knowledge on zinc binding proteins of wheat seed.

In this project, we aimed to determine the effect of environmental conditions on the protein and micronutrient content of the durum wheat seed and to screen the proteome of the wheat seed to identify the proteins with metal-in particular Zn-binding propensity. Protein analyses were carried out on seeds with extreme N and Zn content. Extraction methods available in the literature were modified to obtain reproducible profiles of the total protein content and to separate seed proteins into fractions of different storage protein classes. Extracts were analyzed using polyacrylamide gel electrophoresis and a procedure for detection of Zn-binding proteins was developed. Results showed Zn-binding by Cys rich proteins in the molecular mass range 30 to 50 kDa. The method was extensively tested to confirm the association of Zn with specific proteins, however, if those proteins bind metal in vivo remains to be shown. Microscopic analysis of in situ staining for protein and Zn localization showed preferential staining in the embryo and aleurone layers of the seed and led to determination of the protein profiles from embryo and endosperm tissues, separately. The methods developed during this and further studies, shall be used to do the same screening for different wild-type and cultivated genotypes of wheat.

BUĞDAY DANESİNDEKİ ÇİNKO BAĞLAYAN PROTEİNLERİN TANIMLANMASI

Onur GÖKÇE

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Anahtar Kelimeler: buğday, çinko, dane depolama proteinleri, metal bağlayan proteinler, protein ekstraksiyonu.

Özet

Buğday danesindeki çinko miktarının tarımsal, çevresel ve insan sağlığı açısından önemi vardır. Önceki çalışmalarda, yüksek miktarlarda mikrobesin, özellikle çinko, içeren danelerin daha iyi çimlendiği ve zararlılara karşı daha dirençli oldukları gösterilmiştir. Mikrobesin alım mekanizmaları ve bitkinin gövde ve tohumlarında birikimi üzerine yapılan deneyler, danedeki çinko ve protein miktarı arasında bir bağ olduğunu göstermiş ve daha çok aleuron tabakası ve embriyoda bulunan bazı proteinlerin, çinko depolamada rol oynayabileceğini önermiştir. Danedeki çinko bağlayan proteinler hakkında daha fazla bilgi bulunmamaktadır.

Bu projede, farklı çevresel faktörlerin, durum buğdayı danelerindeki protein ve mikrobesin içeriği üzerinde olan etkilerini bulmak ve buğday danesi proteomunu analiz ederek, metal, özellikle çinko, bağlama eğilimi olan proteinleri adlandırmak amaçlanmıştır. Protein analizleri, aşırı miktarda azot ve çinko içeren daneler üzerinde yapılmıştır. Literatürde mevcut ekstraksiyon metodları, tüm protein içeriğinin tekrarlanabilir profilini elde etmek ve dane proteinlerini, farklı depolama proteini sınıflarına ayrıştırıp çıkarmak için bazı değişiklerle kullanılmıştır. Ekstraktlar poliakrilamit jel elektroforezi ile analiz edilmiş ve çinko bağlayan proteinlerin belirlenebilmesi için bir prosedür geliştirilmiştir. Alınan sonuçlar, molekül ağırlıkları 30 ile 50 kDa arasında olan sisteince zengin bazı proteinlerde çinko bağlandığını göstermiştir. Geliştirilen metot, alınan sonuçlarda çinkonun ilgili proteinlerle ilişkisini doğrulamak için, farklı şekillerde test edilmiştir, ancak bu proteinlerin in vivo metal bağlayıp bağlamadığı gösterilmemiştir. İn situ protein ve çinko boyamaları sonucu, embriyoda ve aleuron tabakasında daha yoğun boyanma görülmüş ve bu, embriyo ve endosperm dokularının protein profillerinin ayrı ayrı incelenmesine yöneltmiştir. Bu ve ileriki çalışmalarda geliştirilen metotlar, farklı yabani ve evcil buğday genotiplerinin analizlerinde kullanılabilir.

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dedicated to those
who have something,
better yet, anything,
to believe in.

Acknowledgments

Where to start?
When did I start?

Sometimes, it happens so fast.
Yet, you felt you had witnessed the time pass,
and you could hold on before you realized
you are irreversibly stuck with yourself,
on the verge of solitute, again, amidst all the innuendo,
lingering, craving without anticipating, ambivalent,
and you are bewildered, my dear,
and you know best to be cynical, you arrogant prick,
and to aggravate,
you set yourself on the eminence of a pretentious angel,
and dare to keep up.

But, lucky of you,
you have, and seems you will:

Zehra Sayers, what can I say my mentor, for you have believed in me and you reached out and held me and molded me. You tought me, talked with me, laughed with me, respected me, cared for me. You shaped my thoughts, my suspicion, my stubbornness and made me. Nothing I say will satisfy; I simply thank you, very.

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I just have these words and not a better combination to be sincere.
I am thankful, and I never forget.

With my deepest regards,
onurgokce

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List of Abbreviations

ABA	Abscisic acid
APS	Ammonium persulphate
BSA	Bovine serum albumin
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
ddH_2O	Double distilled water
DEDC	Dithiocarb
dMT	Durum metallothionein
DTT	1,4-Dithiothreitol
DTZ	Dithizone
DWF	Durum wheat flour
EDTA	Ethylenediaminetetraacetic acid
GA	Gibberellic acid
GFP	Green fluorescent protein
GSH	Glutathione
GST	Glutathione-s-transferase
GPC	Grain protein content
HMW	High molecular weight
IAA	Iodoacetic acid
ICP-OES	Inductively coupled plasma optical emission spectroscopy
LMW	Low molecular weight
MWM	Molecular weight marker
M_r	Relative mobility
MT	Metallothionein
PA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PAN	1-(2-Pyridylazo)-2-naphthol
PC	Phytochelatin
PMSF	Phenylmethylsulphonylfluoride
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulphate
SU	SabancıUniversity

TCEP-HCl	Tris(2-Carboxyethyl) Phosphine, Hydrochloride
TEMED	Tetramethylethylenediamine
UV	Ultraviolet

Chapter 1

Introduction

1.1 Plants and micronutrients

1.1.1 Micronutrients

Micronutrients, such as transition metals like Fe, Cu, Mn and Zn, are essential for growth and development of the living, as they are found in most redox reactions fundamental for cellular processes and in proteins and enzymes for structural and catalytic activities [1]. Shortage of these metals in plants causes deficiency semptoms and growth is reduced [2] and unfortunately, micronutrient deficient soils are widespread throughout the world [3]. Accumulated Zn in the seeds of *Triticum aestivum* L. wheat cultivars was shown to positively effect vegetative growth and grain yield in zinc deficient soils [4, 5, 6].

Micronutrient deficiency is not only a problem of the plant kingdom. It is estimated that over 3 million people are affected by micronutrient such as Fe, Zn and vitamin A deficiencies. These deficiencies also account for almost two thirds of childhood deaths worldwide and developmental problems [7, 8], the reason being related to the dietary habits of developing countries which is based on cheap but low nutritional cereal products rather than animal products of rich micronutrient content [9, 10, 11, 12]. Fortunately,

there are ways to fight this problem, such as food fortification, improving cereal varieties, addition of enhancers for zinc absorption, changing milling procedures and several more, along with combined strategies of them [13].

Therefore, it is beneficial to increase micronutrient, especially Zn, content in the seeds of the wheat for both agricultural and world human health purposes. This can be done by agricultural approaches, such as classical breeding methods and fertilizer application [14, 7], as well as genetic modifications [15].

On the other hand, excess amounts of these essential metals along with nonessential metals such as Pb, Hg and Cd are toxic due to a range of interactions at the cellular and molecular level [16, 17]. Toxicity arises from oxidative damage [18, 19], disruption of protein function [20] and/or genotoxicity[1] [21].

For these reasons, cellular concentrations of these metals must be carefully regulated both to avoid deficiencies and toxicity.

1.1.2 Homeostasis and metal accumulation in plant body and seeds

General overview of mechanisms of metal accumulation has been summarized in Figure 1.1.

Micronutrient metals mentioned above are among the most abundant elements on Earth, but their bioavailability may be limited due to low solubility in oxygenated water and binding to soil particles, therefore, what is abundant cannot be easily taken up from the soil [22]. Focusing on Zn, availability and absorption of Zn from the rhizosphere is affected by several factors including those that are chemical, such as soil pH, redox potential or nutrient

[1] Causing damage to or mutation of DNA.

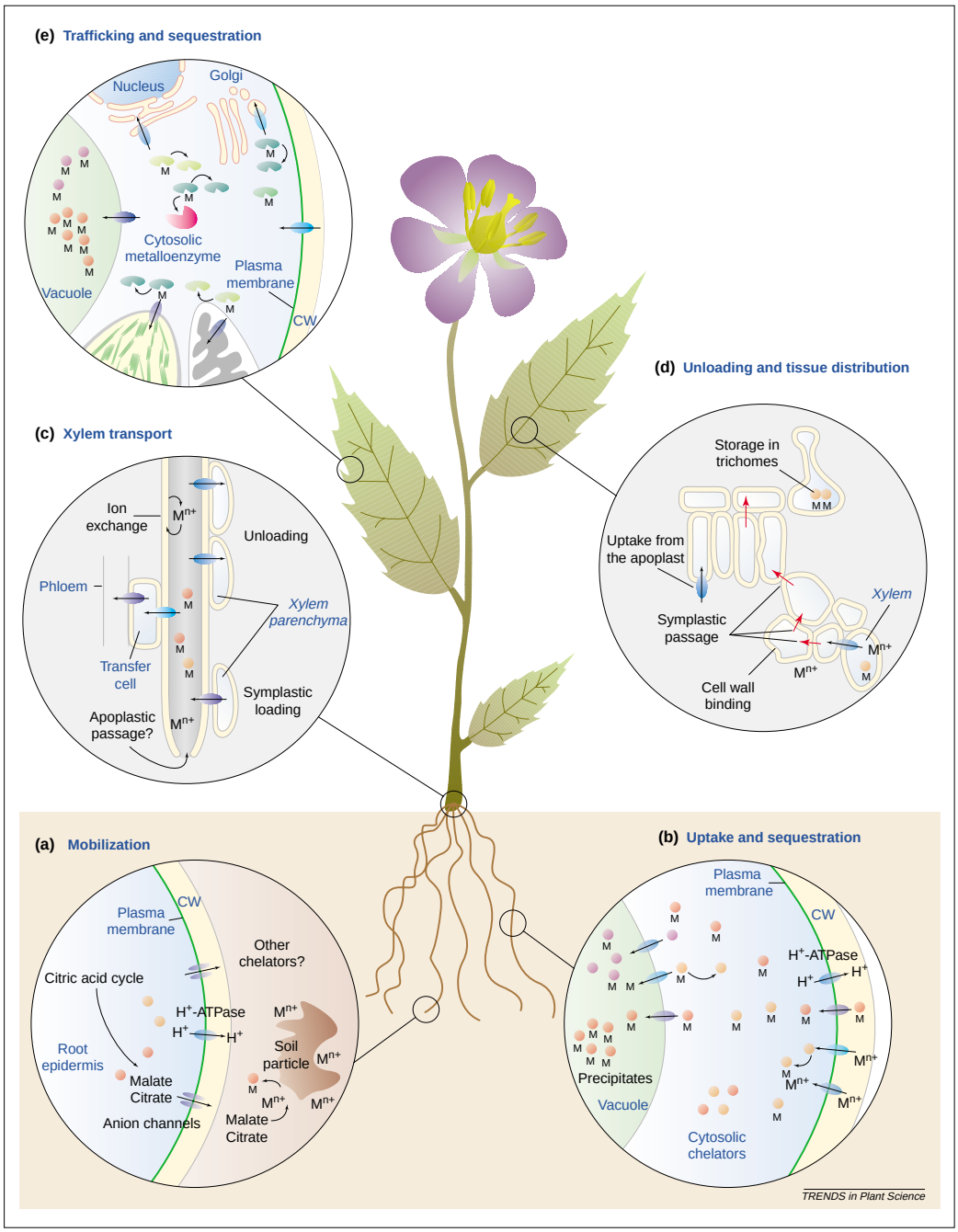

Figure 1.1: Molecular mechanism of transition metal transport [22].

interactions; physical, such as matter content, soil texture or clay content and type; and biological, such as mycorrhizae formation, phytosiderophore[2] release. All these factors work on the root-soil interface [23].

Plants have adapted various strategies to mobilize most of the metal content in the rhizosphere. These include improved root architectures (e.g. increasing root/shoot ratio [24], root colonization by mycorrhizal fungi [25] or bacteria [26]), altering the chemistry of the rhizosphere by changing the pH of the soil or by releasing organic ligands to chelate soil Zn and thus increase the availability.

Mobilized Zn, mainly as Zn^{2+} ion [27], sometimes as Zn-phytosiderophore complex, is uptaken by roots, first by diffusion into root cell walls then into the plasma membrane by the aid of ion transport proteins (Figure 1.2) [1].

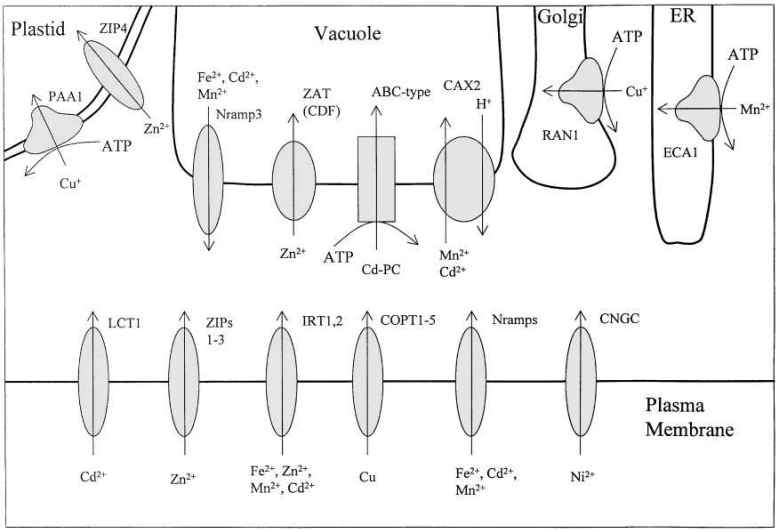

Figure 1.2: Plant metal transporters [1].

Long distance transport of Zn is believed to be faciliated by the xylem stream, because high concentrations of Zn is found in the xylem sap and

[2]Phytosiderophores are nonprotein amino acids that chelate a number of micronutrients.

accumulation of Zn in older leaves even during Zn deficiency suggests that phloem mobility of Zn is low [2], but not impossible [28]. The transportation of ions into the xylem is regulated by membrane transport proteins, however those for metals are not known.

When the metals reach to the tissues in the shoot, homeostasis is regulated by complex mechanisms involving transportation, chelation, trafficking and sequestration into vacuoles [29]. Zn is not found as an ion in the cells, it is chelated and there are various sinks: phytochelatins, metallothioneins, phytic acid, etc. These are discussed in Subsection 1.2.4 on page 17.

1.2 Seed of the wheat

1.2.1 Morphology

Seed of the wheat consists of three major divisions, which are the testa (seed coat) derived from the integument[3], diploid embryo that develops from the zygote of the fusion of the egg cell and a sperm nucleus, and the triploid endosperm emerging from the fusion of the two polar nuclei and a second sperm nucleus (Figure 1.3) [30, 31]. Upon the differentiation during development of the seed new tissues emerge in embryo and endosperm. The tissues of the embryo is out of scope here. Mature endosperm consists of starchy endosperm, aleurone layer, transfer cells and embryo-surrounding region [32]:

Starchy endosperm Largest body of cells in the endosperm that function to accumulate starch, prolamin storage proteins, macromolecules and minerals required for the tissues after germination [34].

Aleurone layer One to several cells thick layer that covers the perimeter of the starchy endosperm which during germination functions in the

[3]The envelope of an ovule

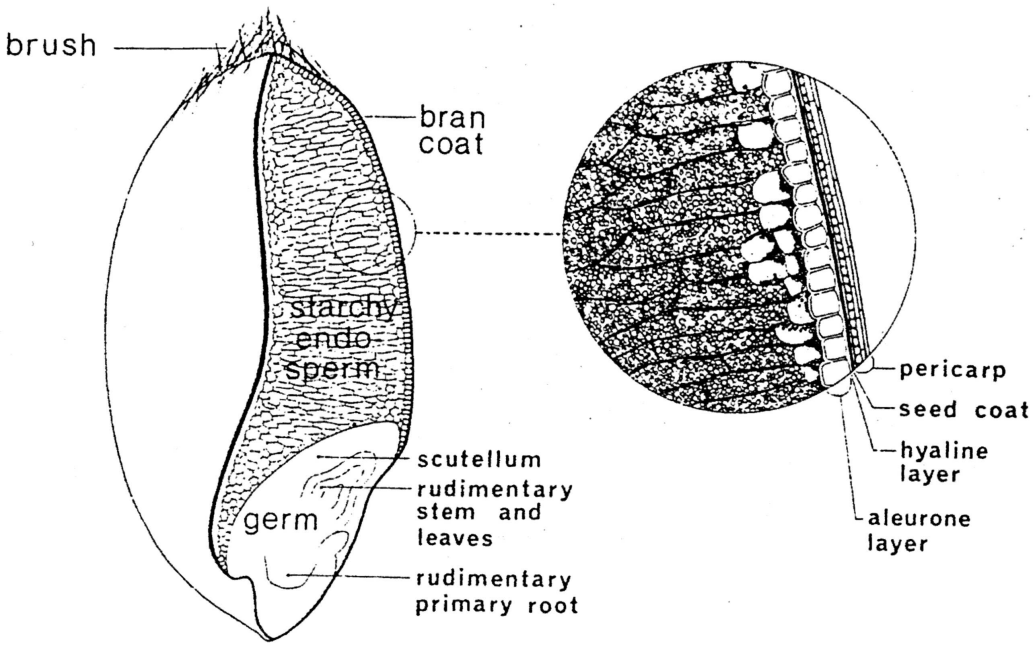

Figure 1.3: Sections of the wheat kernel [33].

mobilization of starch and protein reserves from the starchy endosperm. Aluerone layer is very nutritious. The protein content is remarkably high with amino acid content well balanced. Sulfur containing amino acids (methionine and cysteine) are low when compared to others. More than 80% of minerals like K, Mg, Ca, Fe and Zn in the wheat seed are concentrated in aleurone layer [35]. Cytoplasm of the aleurone cells are filed with organelles, dominantly by protein storage vacuoles, where amino acid building blocks are stored for quick enzyme synthesis [36]. These vacuoles are also the principal storage of minerals in the cereal grain.

Transfer cells Faciliates the transport of amino acids, sucrose and monosaccarites between the endosperm and maternal plant during the development of the seed [37].

Embryo-surrounding Region Although the exact function is not known, it is possible that this tissue provides nutrition to the embryo and provides a barrier and communication zone between the embryo and the endosperm of the seed. These cells have dense cytoplasmic contents, particularly in maize [38].

1.2.2 Development

Whole life cycle of angiosperms has been summarized in Figure 1.4.

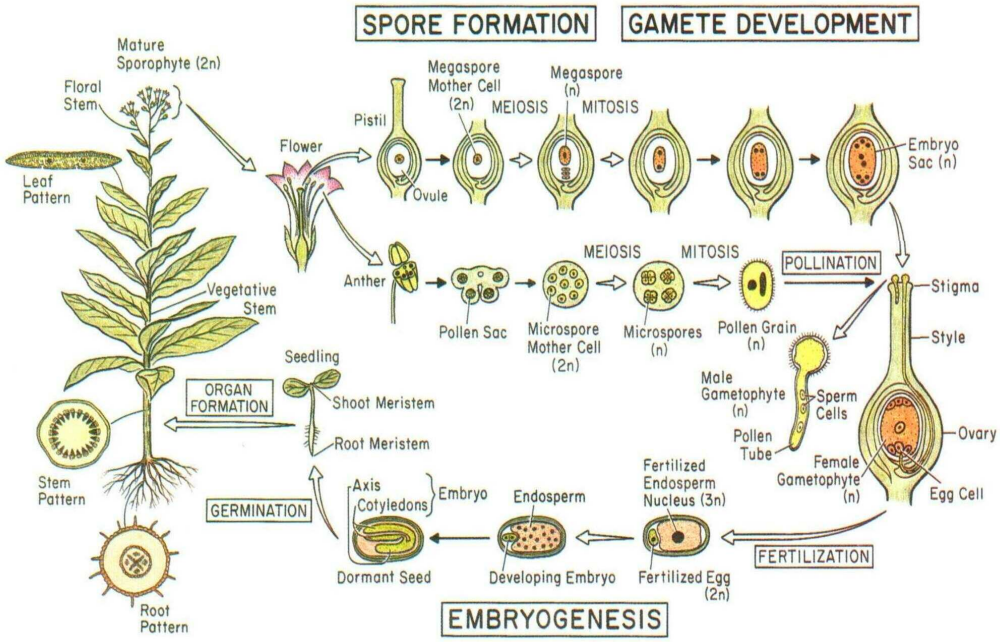

Figure 1.4: Life cycle of angiosperms [39].

Development of the wheat seed, as other angiosperms, starts with double fertilization, where one sperm nucleus fuses with the egg to produce the embryo, while a second sperm nucleus fuses with the two polar nuclei to form the progenitor of the triploid endosperm [40]. Therefore, development of the seed involves embryogenesis where the single celled zygote undergoes complex se-

ries of morphological and cellular changes which results in a developmentally arrested, dormant, embryo [41] and also it involves the differentiation of endospermic tissues to give the mature endosperm. The development requires complex interactions between cells within tissues, as well as interactions between embryo, endosperm and maternal tissues [40].

Embryogenesis starts with morphogenesis, where the polar axis of the plant body is defined by specification of shoot and root apices and embryonic tissues and organ systems are formed. Embryo maturation follows morphogenesis and storage reserves are accumulated. In the last phase of embyogenesis, embryo becomes desiccated and development is arrested until germination [41].

The endosperm development has three general patterns, which are nuclear, cellular helobial and wheat endosperm development is nuclear with following phases: syncytial, cellularization, differentiation and maturation [30, 42, 43].

In the syncytial phase, the primary endosperm nucleus, formed by fertilization, goes through mitosis without cytokinesis, therefore resulting in a multinucleate cell (coenocyte). Cellularization follows until every cell in the endosperm becomes uninucleate (Figure 1.5). Cellularization and differentiation are closely integrated processes in terms of timing and place. They occur simultaneously. Those nuclei close to the maternal tissues differentiate to become transfer cells, those close to the embryo become embryo surrounding cells. Peripheral cells of the first periclinal division of the alveolar nuclei become aleurone, daughters without preprophase bands [44] become starchy endosperm. [45, 34].

Cellularization and differentiation is followed by maturation of the tissues which involve cellular expansions and reserve depositions. During seed

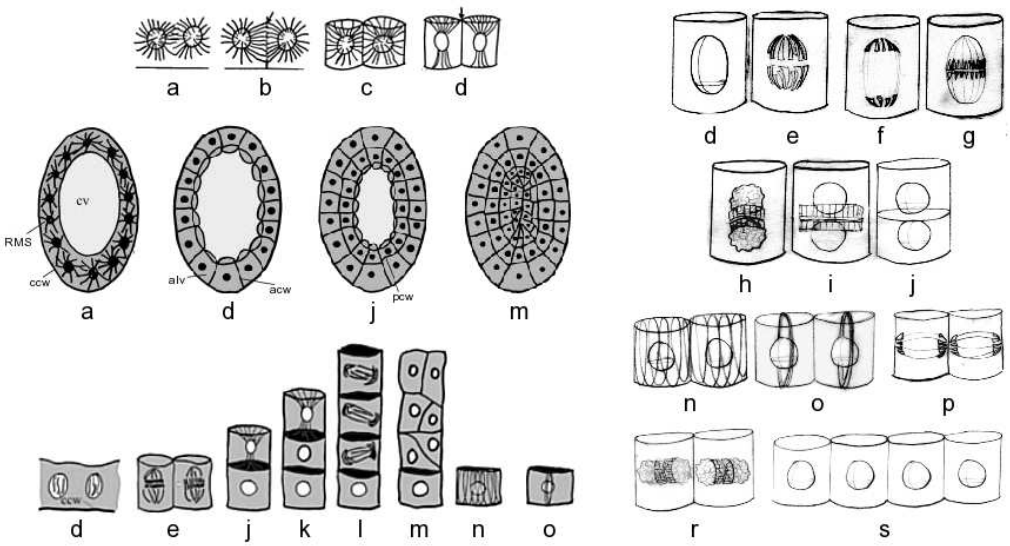

Figure 1.5: Cellularization and differentiation of endosperm coenocyte of cereals. **(a–d)** Anticlinal cell wall formation: (a) Cellularization is initiated by the formation of radial microtubule system (RMS) on all nuclear surfaces. (b) RMSs extend and initially overlap, soon forming cytoplasmic phragmoplast that deposit anticlinal cell walls (acw). (c) Partial anticlinal cell walls are formed around each nucleus. (d) Walls extend centripetally toward the central vacuole (cv) by the aid of adventitious phragmoplast formed by a canopy of microtubular arrays extending from upper pole of endosperm nuclei. Endosperm alveoli is formed (al). **(d–j)** Periclinal division in endosperm alveoli: (e) Mitotic spindles in metaphase, periclinal orientation. (f) Mitotic spindles in anaphase, periclinal orientation. (g–i) Funtional interzonal phragmoplast is formed at mitotic interzone, separating the daughter nuclei. Periclinal endosperm cell wall (pcw) is deposited. (j) Second layer of cells are formed. Peripheral cells have preprophase bands (PPB). **(j–m)** Endosperm cell file development: (j–l) Process repeated three times to have four layers. (l) Mitotic divisions in the endosperm cells that lack PPBs at random division planes. (m) Starchy endosperm cells are formed. **(n–s)** Expansition of the aleurone layer: (n) Two neighboring aleurone cells with hoop-like cortical arrays. (o) Future of division plane is determined by PPBs. (p) Mitotic spindle axis. (r) Interzonal phragmoplast formation in the anticlinal plane. (s) Four aleurone cells are formed [32, 45]. (ccw: central cell wall.)

development, endosperm cells synthesize storage polymers that are largely insoluble and have low osmotic activity. Primary stored polymer in the starchy endosperm is starch. Storage proteins accumulate in the grain to supply nitrogen and sulphur for the embryo [46].

Cereal seed storage proteins are produced by the secretory pathway and deposited in discrete protein bodies. Wheat prolamins (gliadins) are synthesized on the rough endoplasmic reticulum and accumulate in membrane-bound protein bodies. Two mechanisms have been observed in the formation of protein bodies in different cereals. After their synthesis in rough endoplasmic reticulum (ER), either proteins are deposited within the lumen of rough ER, such as prolamins of maize, rice and sorghum or proteins are packed into protein bodies in golgi apparatus, such as rice glutelins. In wheat, the mechanism of gliadin deposition in protein bodies also makes use of the golgi apparatus [47]. Among the factors that affect the protein composition of the seed is the availability of nitrogen and sulfur. When nitrogen is sufficient enough, varying sulfur supply causes changes in the abundance of S-rich or S-poor proteins [48].

During late maturation, till the end of the development, starchy endosperm cells undergo programmed cell death accompanied by an increase in nuclease activity and internucleosomal fragmentation of nuclear DNA, induced by ethlene. Death of the starchy endosperm tissue ensures and is required for rapid hydrolysis and mobilization of storage reserves during germination. This process is regulated, so that premature death of starchy endosperm cells can be avoided, which if not, would result in a limitation of storage reserve synthesis [30].

1.2.3 Germination

Germination is the group of events that start with imbibition by quiescent dry seed and end with the elongation of the embryonic axis. The visible sign of the completion of the germination is the penetration of the structures surrounding the embryo by the radicle. Later events as the mobilization of major storage reserves are associated the growth of the seedling [49]. Various phases and major events are summarized in Figure 1.6.

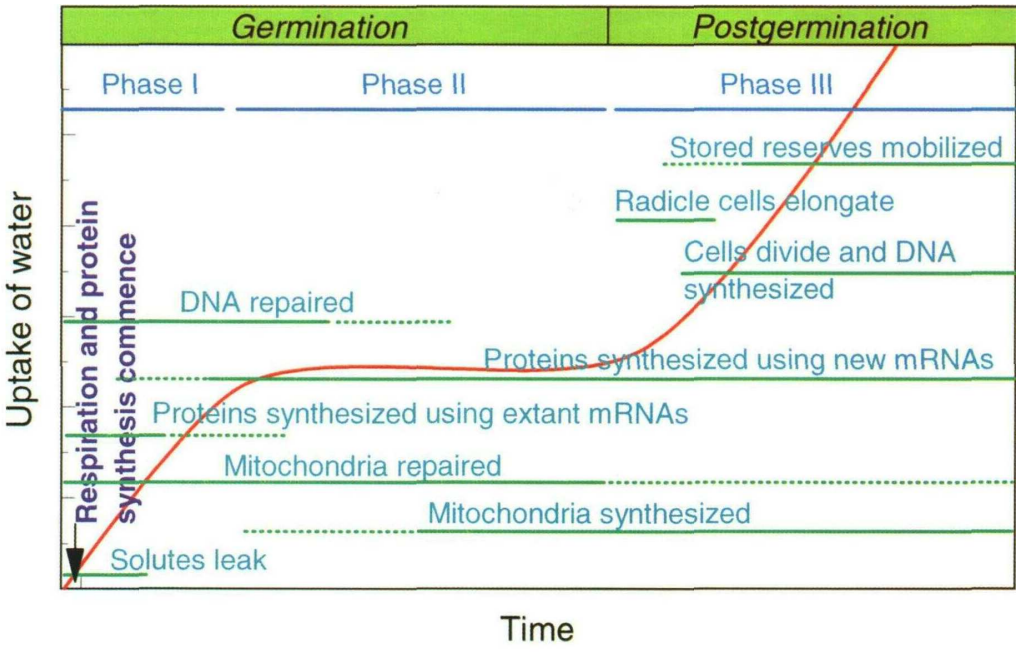

Figure 1.6: Phases of germination and major events that take place [49].

As the dry seed is soaked in water, the imbibition causes termporary structural perturbations to membranes and this leads to immidiate and rapid leakage of solutes and low molecular weight substances, which may be explained by the transition of membrane phospholipid components from gel phase to hydrated liquid-crystalline state [50].

With imbibition, dry seed resumes its metabolic activity. The structures

and enzymes that are required for the initial metobolism are present within the dry seed. Respiratory activity is restored within minutes of water contact after poorly differentiated mitochondria, already present in the quiescent seed, go through rapid development. Oxygen consumption rate declines until the end of germination, but increases immidiately after [51].

Protein synthesis is recovered upon imbibition since all of the components required are present in the mature dry embryos, except for polysomes, which are assembled from the ribosomes after rehydration. Initial protein synthesis is dependent on the extant ribosomes, however new ribosomes are produced and used within hours of initial polysome assembly [52]. Preformed mRNA is also present at the mature dry seed and some of these residual mRNAs are associated with previous developmental processes [53] and may be used in early germination. Those mRNAs that are only residual and will not be used during germination degrade rapidly [54, 55], whereas those that translate the proteins required in early germination are used, but replaced gradually by newly synthesized identical mRNAs and transcription becomes more and more dependent on the fresh copies [56]. Among those residual transcripted genes, mRNAs translating for germin, E_m (early methionine-labelled) and E_c (early cysteine-labeled; gene encoding Zn binding metallothionein [57]) are present [58].

Imbibition triggers the embryo to release gibberellic acid (GA), which in turn diffuses to the scutellum and the aleurone layer, and induces the synthesis and secretion of numerous hydrolytic enzymes to breakdown the polysaccharides, storage proteins, lipids and nucleic acids in the starchy endosperm. The products are relayed back to the embryo via the scutellum (Figure 1.7) [34, 36]. These hydrolases also breakdown storage proteins in protein storage vacuoles of the aleurone cells. During these processes, ab-

scisic acid (ABA) counterworks against GA [59]. After mobilizing all their content, aleurone cells go through programmed cell death [36].

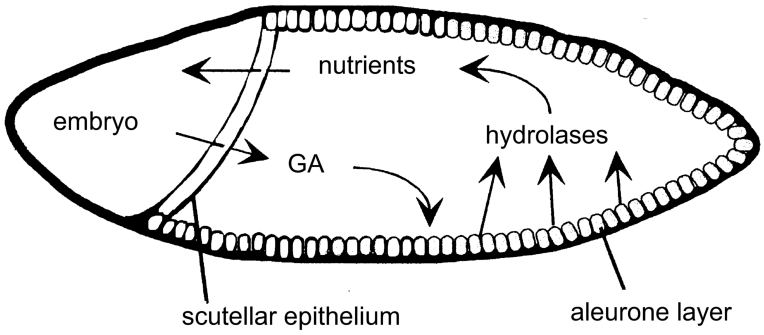

Figure 1.7: Mobilization of stored nutrition in starchy endosperm [34].

Among the factors that effect germination, protein content of the seed should be noted. Comparing germinating seeds of high and low protein content, it was shown by Ching *et al.* [60] that high protein content resulted in a faster rate of seedling growth and a higher yield of product.

1.2.4 Proteins of wheat seed

Classification of the proteins of cereal seeds has followed various schemes over the years. Initial, and still valid, classification by Osborne [61], was based on the solubility of the proteins and they were grouped according to their solvents: albumins (water), globulins (dilute saline), prolamins[4] (alcohol-water mixtures), and glutelins[5] (dilute acid or alkali or SDS solution). Some proteins are grouped further according to their sedimentation constants, such as 2S albumins [62], 7S and 11S globulins. More recent classifications grouped proteins by their functional roles as storage, structural and biologically active proteins by Fukushima in 1991 [63] and later as storage, structural/metabolic

[4]Prolamins of the wheat are called gliadins.
[5]Glutelins of the wheat are called glutenins.

and protective proteins by Shewry *et al.* in 2002 [64]. New ways of classification emerge, one of them being based on structural and evolutionary relationships [65]. With this classification, over 50% of seed proteins are classified within prolamin and cupin superfamilies.

Because of their dominance in seeds and their agricultural importance, most research has been conducted on cereal storage proteins. These proteins, along with metal binding proteins and peptides of the seed, will be introduced here because of their relevance for this thesis.

Storage proteins

Seed storage proteins have some distinct characteristics. During the development of the seed, they accumulate in high amounts in protein bodies, to be used later during germination. These proteins are synthesized only by the endosperm tissues and lack any other function other than storage. Since they are used for nitrogen storage, they are rich in asparagine, glutamine, arginine and prolamine [66].

Prolamin superfamily

Prolamins are major storage proteins in the endosperm of cereals and form the major components of gluten protein fraction in the seed. Their molecular weights are between 10 kDa and 100 kDa. These proteins have two common structural features: the presence of distinct regions or domains adopting different structures to each other, possibly from different origins; the presence of repeated blocks of one or more short proline and glutamine-rich motifs, or repeated blocks enriched in residues such as methionine. The prolamins of Triticeae (wheat, barley and rye) have been divided into three groups, sulphur-rich (S-rich), sulphur-poor (S-poor) and HMW, based on

their sequences, which can further be classfied based on the amino acid composition [64].

S-rich prolamins are the major group of prolamins in Triticeae, most having molecular weights between 36 kDa and 44 kDa. They have a similar basic structure with proline-rich repeats at the N-terminal domain and a C-terminal that has most, if not all, of the cysteine residues. In some proteins, repeats of the N-terminal domain may be preceeded by a short unique sequence. There are three types that belong to this class of proteins, which have structural differences when looked in detail: γ-type, α-type and aggregated type (LMW subunits of glutenin). These proteins are thermally stable [67].

S-poor prolamins lack cysteine residues and therefore cannot form oligomers or polymers. Molecular weight of these proteins are between 44 kDa and 78 kDa. They have β-turn rich structures. ω-gliadins are the corresponding proteins in wheat [67].

HMW prolamins are the HMW subunits of wheat glutenin and have two types, x-type (M_r 83–88 kDa) and y-type (M_r 67–74 kDa). They have a clear domain structure, each type consisting of non-repetitive regions forming the N-terminal domain and the C-terminal domain and a region of repeating motifs between these termini. Most of the cysteines are in the non-repetitive domains; three in x-type, five in y-type N terminal domain and one in C-terminal domain of both x and y-type. The repeating motifs are a hexapeptide (Pro-Gly-Gln-Gly-Gln-Gln) and a nonapeptide (Gly-Tyr-Tyr-Pro-Thr-Ser-[Pro/Leu]-Gln-Gln), found in both types of glutenin, and a tripeptide (Gly-Gln-Gln) for the x-type only. Structural of glutenin are predicted to be α-helical at the terminal domains and β-turns in the central repetitive region [67].

Cupin superfamily

Cupin family is a superfamily of prokaryotic and eukaryotic proteins, having a common double-stranded β-helix (jelly-roll) barrel structure that is thought to come from a prokaryotic ancestor. They have a two-motif sequence Pro-Gly-$(xxx)_5$-His-xxx-His-$(xxx)_4$-Glu-$(xxx)_7$-Gly and Gly-$(xxx)_5$-Pro-xxx-Gly-$(xxx)_2$-His-$(xxx)_3$-Asn which are separated by 15–50 amino acids. Proteins belonging to this family are generally single or double domain, each domain having the common structure, however multidomain proteins with a single cupin domain, such as some transcription factors exist [68, 69].

Germin and germin-like proteins are from the single-domain proteins of the cupin superfamily which are extensively used during development and germination. They have metal binding capacity. Germins have different functions and uses, including oxalate oxidase enzyme in wheat, structural proteins in barley and receptors in barley [70].

Globulins, proteins of interest of this section, are double domain bicupins, deficient in cysteine and methionine. Depending on their sedimentation coefficients, globulins are grouped in two types, which are 7S vinicilin-type and 11–12S legumin-type. 7S type is related to the vicilin proteins of legumes and proteins of this type are typically trimeric with M_r values approximately between 150 kDa and 190 kDa. They go through post-translational processing (proteolysis and glycosylation), therefore their detailed subunit compositions show varience. In wheat, oat and barley, proteins of similar structure and properties, but with limited sequence similarity are found in the embryo and the aluerone layer [64, 71].

11S type globulins are related to the legumins of legumes. Tricitin is the corresponding protein of the wheat which make about 5% of the total

seed protein. They are found in the starchy endosperm, forming dimeric structures with a large (M_r 40 kDa) acidic polypeptide chain linked to a small (M_r 22–23 kDa) basic chain by a single disulphide bond [72, 71].

Metal binding proteins and metal storage

Metallothioneins

Metallothioneins (MTs) are described as low molecular weight (4–8 kDa), cysteine rich, gene encoded proteins. High number of cysteine residues of these proteins, allow binding of metals through mercaptide bonds. Typically, metallothioneins have two metal binding, cysteine rich domains, linked with a hinge region. They are classified according to the arrangement of Cys residues into three classes. Class I MTs are a group containing 20 highly conserved Cys residues based on mammalian MTs, lacking aromatic amino acids, and are commonly seen in vertabrates. These can coordinate seven divalent or twelve monovalent metal ions in two clusters [73]. In animals, MTs function in the Cu and Zn homeostasis and protection from Cd toxicity [74]. There are four different isoforms of Class I MTs that have been described in mammalians; MT-1 and MT-2 are expressed in all organs, where MT-3 is more abundant in the brain and MT-4 mostly in certain stratified tissues [75].

Class II MTs are MTs without strict arrangements of Cys, constituting all MTs of plants, fungi and some nonvertebrate animals. As more MT genes were isolated from plants, Class II MTs were further grouped into different types, based on amino acid sequence similarity [73]. Plant Type 1, 2 and 3 MTs all have two cysteine-rich domains, separated roughly by 40 amino acids including aromatic residues, in contrast to MTs from other organisms where the hinge region is less than 10 amino acids long and lack aromatic residues. Type 1 has six Cys-xxx-Cys motifs equally distributed among two

domains. Type 2 is slightly different than Type 1 in terms of Cys distribution and in term of the diversity of the hinge region among species. Type 1 and 2 MTs were first classified by Robinson *et al.* [76]. Type 3 MTs are expressed during fruit ripening and have slight seqential differences to Type 1 and 2. Type 4 includes the wheat E_c protein, which is the first identified plant metallothionein. This protein is expressed during embryogenesis and bind Zn [77]. Unlike to other plant MTs, Type 4 has three domains with each containing 5 or 6 conserved cysteine residues, separated by 10-15 amino acids [73]. The expression of MTs are induced in plants by Cu and to a lesser extend by Cd and Zn [78, 79, 80].

Phytochelatins are classified as Class III MTs, but these peptides are synthesized rather than gene encoded.

Phytochelatins

Phytochelatins (PCs) are polypeptides that are formed only by Glu, Cys and Gly amino acids and are classified as class III MTs which play a key role on metal homeostasis and heavy metal detoxification. The general structure has repetitions of γ-Glu-Cys dipeptide followed by a terminal Gly. These repetitions are usually in the range of 2 to 5, sometimes up to 11. Structural resemblence of phytochelatins to glutathione (GSH) tripeptide suggested, and this has later been confirmed, that phytochelatins are synthesized from GSH by phytochelatin synthase enzyme [81], which is shown *in vitro* [82] and in studies with mutants [83] to be activated by heavy metal ions (Figure 1.8). Variants have been identified in some plant species, that had the same repeating dipeptide, but ended with a different terminal amino acid and synthesized not from GSH, but from related compounds. Five major families are identified with following structures [84]:

1. (γ-Glu-Cys)$_n$-Gly (phytochelatin)

2. (γ-Glu-Cys)$_n$-β-Ala (homophytochelatin)

3. (γ-Glu-Cys)$_n$

4. (γ-Glu-Cys)$_n$-Ser (hydroxymethyl-phytochelatin)

5. (γ-Glu-Cys)$_n$-Glu

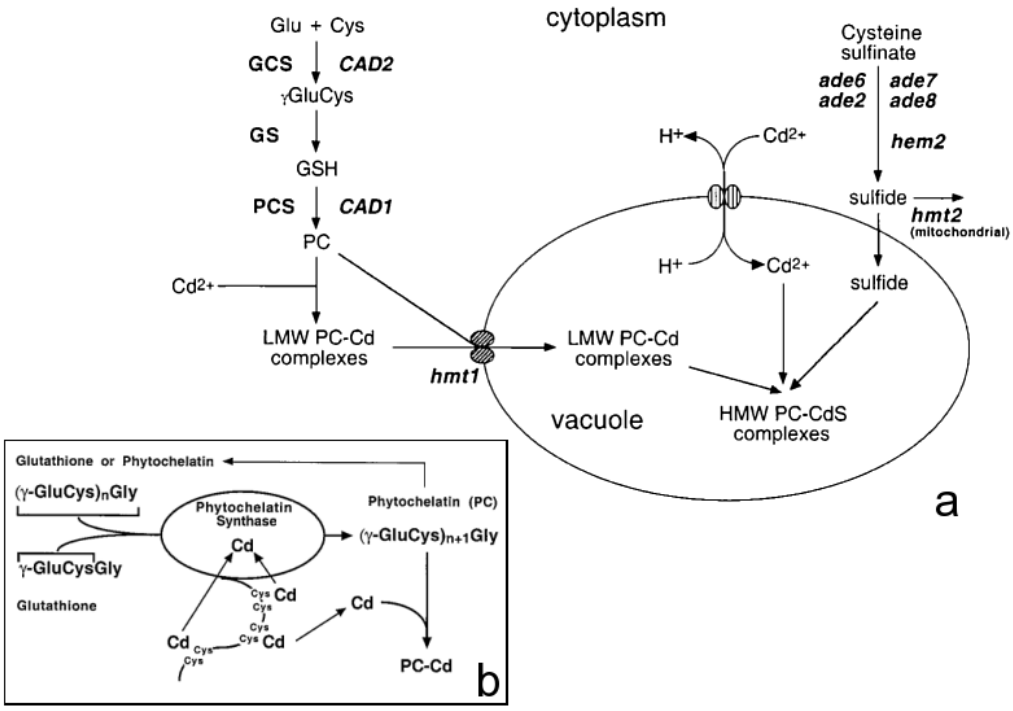

Figure 1.8: Synthesis of phytochelatins. (a) Synthesis of phytochelatins, sequestration of Cd and deposition in the vacuole. (GCS: γ-glutamyl-Cys synthetase, GS: GSH synthetase, PCS: PC synthase) (b) Model mechanism of PC synthase activity. Cys rich C-terminal of the enzyme works as a heavy metal sensor, relaying metals to the catalytic domain at the N-terminal [85].

Phytochelatins can bind heavy metals such as Cd, Hg, Cu, Zn, Ni and Ag via thiolate coordination and their per Cys residue metal binding capacity is

higher compared to those of MTs [86]. The exact mechanism of regulating the biosynthesis of the phytochelatins is not known, but various models exist [85].

Other proteins

Among proteins that bind metals for storage and detoxification, ferritins should be noted. These are proteins that play an important role in the iron metabolism as they are able to sequester many iron ions. They have a ubiquitous distribution among species and three-dimensional structure is highly conserved. All ferritins have 24 subunits arranged in 432 symmetry[6] that give a hallow shell of 80 Å diameter which can store up to 4500 Fe(III) atoms [87]. Although not reported in plants, it is showed that animal ferritins are able to bind metals other than Fe, such as, Cu, Zn, Cd, Pb, Be and Al [17]. In transgenic rice with soybean ferritin, Zn accumulation was noted to be higher, although the mechanism is not known [88].

As noted earliar, cupin superfamily proteins are also able to bind metals because of their structure [69], however it seems to be more for catalytic purposes rather than storage.

It was also shown in various plants that the expression of heat shock proteins (HSPs), that are generally expressed more in organisms which are grown at temperatures higher than their optimal growth temperature, increased in response to heavy metal stress [89].

Small molecular weight metal chelators

Additional to the proteins and polypeptides mentioned above, several low molecular weight compounds play a significant role in metal homeosta-

[6]achiral octahedral symmetry

sis. These include some organic acids (e.g. citrate, malate, oxalo acetate), some amino acids (e.g. histidine) and their derivatives (e.g. nicotianamine, mugineic acids) and phosphate derivatives (e.g. phytate) [17].

Phytates are mono to dodeca anions of phytic acid (myo-inositol-1,2,3,4,5,6-hexakis(dihydrogen phosphade)) and they constitute about 0.5–2.0% by weight of cereal seeds, being concentrated at the aleurone. Various physiological roles are described, as phosphate and inisitol source, as reactive phosphoryl groups source, as energy source and possibly as source for cations [90]. A heterometallic Mn/Zn-phytate complex has been presented as a model for grain metal storage [91].

It was shown also that phytates interact with proteins, although the nature is not known. It is possible that the interaction is electrostatical, since, depending on pH, up to 12 hydrogens may dissociate from phytic acid, yielding phytates of different degree of protonation and since proteins are also charged molecules, especially at a lower pH than the isoelectric point of proteins, where lysine, histidine and arginine residues can be positively charged. Since polyvalent cations also interact with phytates, introducing such cations decrease the interaction between phytic acid and the protein, forming phytic acid-mineral-protein complexes [92]. This interaction may also be present in the cereal and legume seeds [93].

Although protective roles of phytic acid are known, still it is not a good nutritional compound as it is a strong chelator for minerals such as Zn, Fe, Ca and Mg and causes a decrease in the bioavailablity of these elements [94].

1.3 Metal binding proteins

Understanding the structural chemistry of the metals, metal binding sites of proteins and the chemistry of the binding itself is important, not only be-

cause it provides a better understanding for the workings of metal storage, metalloenzymes, transcriptions factors, etc., but also, this knowledge develops the intuition to understand about one-third of all proteins (that require metals for their structure and function), and to design novel metal binding peptides.

Following section will provide some theoritical basis for the experimentation conducted during this research.

1.3.1 Protein chemistry with respect to metal cations

Generally speaking, protein sites with bound metal have following functions [95]:

1. structural: metal is required for the conformation;
2. storage: uptake, binding and release of metals in soluble form;
3. electron transfer: uptake, release and storage of electrons;
4. dioxygen binding: for O_2 coordination and decoordination;
5. catalytic: substrate binding, activation and turnover.

Complexing power of a metal ion with a metal-binding site is dependent on its polarizing power, which is the ratio of charge to ionic radius. As the metal ion polarizability increases, high density positive charge also increases, resulting in stronger interaction between the metal and the protein. Looking at solely the properties of the metals, some things may be presumed about the binding site, however proteins also influence metal site geometry and activity [96].

Proteins coordinate metal ions with nitrogen, oxygen and sulfur. Side-chain carboxylate, sulfur and imidazole groups dominate the metal coordination in proteins, but in some sites, main-chain carbonyl oxygens are also used. First row transition metal ions, except for Zn^{2+}, are subject to ligand field stabilization. Since Zn^{2+} is a d^{10} metal ion (valence d shell has 10 electrons), it is not stabilized to be coordinated in a octahedral geometry, therefore Zn^{2+} ion-binding sites almost always have a distorted tetrahedral geometry, but at some instances, it might have a 5 or 6 coordination geometry. Because of its electron configuration, Zn^{2+} does not have a biologically relevant redox activity, however it acts as an electrophilic catalyst that stabilizes negative charges during an enzyme-catalyzed reaction. Usually, catalytic Zn^{2+} ion is coordinated by His residues, whereas it is coordinated by Cys residues for structural purposes [97, 95].

The metal-ligand bond is dependent on the detailed nature of the valence orbitals of ligands, as well as the effective nuclear charge and coordination number and geometry of the metal ion [95]. Recognition of a transition metal by a protein is faciliated by discrimination of ionic size, charge and chemical nature of the metal by the binding site. These factors are summarized as the hardness/softness of the metal. One binding site for a cation would not be suitable for another cation, as the chemical composition of the binding site is usually optimal for a certain cation. Hardness of the binding site needs to be complementary to the hardness of the cation for the optimum binding. A number of interactions, additional to the one above, work on the protein-metal ion recognition, such as electrostatic, molecular orbital and entropy effects [97].

Abundance of polarizable cysteine thiolate ligands in MTs, gives the proteins a high affinity for soft d^{10} metal ions such as Zn^{2+}, Cd^{2+} and Cu^{+} and

naturally occuring MTs are isolated with these ions [75].

Metal binding sites are usually located inside cavities and crevices of the protein, making the site solvent inaccessible, which in turn results in a low dialectric constant enhancing the metal-protein ligand interactions. This favors inner-sphere metal binding. Binding site selectivity appears to be anticorrelated with the natural abundance of a metal in the living system. For instance, Mg-binding sites are not very specific for Mg, which is the most abundant mineral in body fluids. As a result, these sites are weakly protected against other metals. However, binding sites for elements found in minute quantities have evolved to become very specific, in order to selectively bind the required metal ion, eliminating binding of other cations of higher concentrations [98].

1.3.2 Metal and metal bound detection

Monitoring if a protein binds metals involves specific approaches depending on the protein in question. The ideal approach would be purification of the specific protein from the native source with the bound metal, followed by biochemical and biophysical characterization of the holo-protein (metal containing protein) using methods mentioned under "Metal Detection" heading (Page 25). However, this approach requires prior identification of the protein and the metal. In cases where metal-binding proteins are not clearly identified, total protein extraction is carried out wherever possible under conditions that would preserve the native state. Different techniques are then applied to analyze the total extract and identify metal containing fractions. In the cases where protein solubility is severely limited, extraction is carried out under denaturing conditions and metal-binding is tested on apo-protein separated by different techniques.

Bioinformatics should also be used during such a research as some theoritical predictions might save time [99].

After crude fractionation, proteins may be separated from one another and purified using chromatography. Different liquid chromatography applications, including size exclusion, ion exchange, affinity and reverse phase chromatography may be used. The principles of these methods and example metal binding protein applications for each method have been reviewed by de la Calle Guntiñas *et al.* [100]. Immobilized metal affinity chromatography has also been used for the purpose [101]. The use of chromatography systems like fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC) improves the results.

Depending on the setup, structure or the purity of the protein, there are various electrophoresis methods of separation. These are SDS and native polyacrylamide electrophoresis on slab gels or in capillaries [102], two dimensional electrophoresis techniques, such as isoelectric focusing (IEF) [103] and immunoelectrophoresis [104], affinity capillary electrophoresis [105], etc.

Metal detection

Metals can be detected using a variety of ways. The most straight-forward method is staining. There are some colorimetric and fluorometric indicators that chelate metals. Such chemicals, that the wavelength of absorbance or emittance chage upon metal binding, include dithizone (DTZ), 1-(2-thiazolylazo)-2-naphtol (TAN), 1-(2-pyridylazo)-2-naphtol (PAN), tetraphenylporphin (TPP), etc. and some assays are described making use of these stains [106, 107, 108]. Such indicators have also been used to determine the localization of heavy metals in various organisms and tissues; e.g. blood [109, 110, 111], pancreas [112], algae [113], yeast [114], and wheat

seed [115]. Results acquired by these indicators can be assessed visually, or quantitively, by making use of spectrophotometry and fluorometry.

Spectroscopy based methods are more accurate and directly detect for the metal, without the need of an indicator. Some of methods that can detect and identify metals are synchrotron X-ray fluorescence, particle induced X-ray emission, flame atomic absorption spectroscopy, inductively coupled plasma mass spectrometry and optical emission specrometry and the list can be exhausted [116].

Radioactive isotopes of heavy metals may also be used for detection, however, although sensitive, these methods are hazardous.

Metal detection may be done on protein solutions, for instance, on fractions from chromatography, on SDS gels by sychrotron X-ray fluorescence [117], on solid surfaces by reflective synchrotron X-ray fluorescence [118], on PVDF membranes where the proteins are blotted by autoradiography [119, 120, 103], on native gels by laser ablation-inductively coupled plasma-mass spectrometry [121].

Since the metal has an effect on the conformation of the protein, spectroscopic methods, such as circular dichroism (CD), nuclear magnetic resonance (NMR), near-infrared spectroscopy, etc., may allow to monitor if the protein is bound to a metal or not. However, prior data on the protein is required to assess, as these methods are based on comparisons between different conformations of the proteins. This way of metal detection is indirect.

Combined methods

Techniques mentioned above are the most basic forms. High-throughput and sensitive results are acquired by combining several methods. So called in-line hyphenated techniques, that are coupled separation and detection

techniques, increase the accuracy and precision of the measurements. The setup of these techniques allow the sample, for instance, to be separated by HPLC or capillary electrophoresis, then, as the sample is separated, quantify the metal concentration and identify the proteins, sequentially and continuously [122, 123].

1.4 Purpose of study

It is of great importance to be able to maximize stored essential micronutrient amount in cereals, especially in wheat, for the reasons discussed above.

In this study, proteome of the wheat seed (specifically of durum wheat) is screened for proteins which have metal binding properties. Balcalı2000 wheat cultivars are grown under varying environmental Zn and N concentrations. Wheat plants and seeds are analyzed for Zn, N and P content and correlations with environmental conditions are determined.

Total and fractionated proteins are characterized by PAGE analysis and methods are developed for detection of Zn-binding/chelating by proteins that are separated on polyacrylamide gels or on those that are blotted onto membranes. Reliability of the method for determining metal storage proteins and for screening different wild-type and cultivated wheat genotypes is assessed through control experiments.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Equipment

For the detailed list of chemicals and equipment that has been used during this project, please refer to Appendix B.2 and B.3.

2.1.2 Solutions

For the recipes of solutions that are mentioned only by name, please refer to Appendix B.1.

2.2 Methods

2.2.1 Greenhouse trials with plants growth under different zinc and nitrogen concentrations

Different zinc and nitrogen concentrations were applied to the durum wheat genotype "*Triticum turgidum, cv.* Balcalı-2000" for the greenhouse trial. These concentrations were 0.5 ppm, 8 ppm, 24 ppm and 24 ppm with foliar $ZnSO_4$ application for zinc and 75 ppm, 225 ppm, 675 ppm and 675 ppm with foliar urea application for nitrogen. The basal treatment were the same

for all pots (Table 2.1). The pot placement and trial setup is summarized at Table 2.2.

	Element	Chemical	Dosage
Basal	Fe	Fe-EDTA	5 ppm
	P	KH_2PO_4	100 ppm
	S	K_2SO_4	20 ppm
Trial	N	$Ca(NO_3)_2{\cdot}4H_2O$	varying
	Zn	$ZnSO_4{\cdot}7H_2O$	varying

Table 2.1: Applied nutrition and the dosages.

Zinc Concentration (ppm)	Nitrogen Concentration (ppm)			
	75	225	675	675+spray
0.5	6864	6879	6894	6909
	⋮	⋮	⋮	⋮
	6868	6883	6898	6913
8	6869	6884	6899	6914
	⋮	⋮	⋮	⋮
	6873	6888	6903	6918
24	6874	6889	6904	6919
	6875	6890	6905	6920
	6876	6891	6906	6921
24+spray	6877	6892	6907	6922
	6878	6893	6908	6923

Table 2.2: Greenhouse trial setup. Sample numbers for each pot are shown. Seeds, protein extracts or any material derived from these pots are coded with the same number.

The following steps were taken as preparations for the trial:

1. All the equipment, including pots, were washed first with dilute HCl, then rinsed with distilled water.

2. Soil was weighed so that each pot had 3 kg of it.

3. Basal and trial nutritions, as indicated in Table 2.1, were applied to the soil accordingly and mixed thoroughly.

4. 10 to 12 seeds were planted to each pot.

After sowing, the pots were watered on a daily basis, as required. Once the germination was complete, plants were thinned to have 6 to 7 plants at each pot. After about one month, foliar nutrition applications were started. For zinc, 0.2% $ZnSO_2$ solution, and for nitrogen, 0.5% urea solution were sprayed on each corresponding plant until started dripping. The nutrition was applied during the evening and outside the greenhouse to avoid contamination. Plants were carried back to the greenhouse only after the leaves were totally dry. Both nutritent solutions had 0.01% Tween to eliminate the surface tension and thus maximize wetting. This foliar application was repeated 3 more times, until the spikes emerged.

Dosage of basal treatments that were applied before sowing was only adequate for the first two months. As the plants were to continue their growth and as senescence on old leaves were observed, additional nutrition was applied. Each pot received 50 ppm more phosporus (KH_2PO_4) and additional one third of the initial dosage of nitrogen ($Ca(NO_3)_2{\cdot}4H_2O$).

Sister spikes were not removed during the trial. The plants were harvested when all of the spikes turned yellow. Full spikes, empty spikes and the plant bodies were counted and placed in the oven at 45°C in paper bags for complete dehydration. After the removal of residual water, to determine dry weights, spikes, bodies and seeds were weighed.

During the growth, plants were invaded with bugs for several times. 0.2% Didifos 55 EC Pesticide was sprayed on the leaves.

2.2.2 Spectrophotometry

For absorbtion measurements, spectrophotometers were used. Spectra were recorded in the near UV–visible light range or measurements were taken at specific wavelengths. Hellma QH or QS quartz cuvettes were used for measurements in the UV range (380 nm–200 nm), whereas Kodak plastic cuvettes were used for measurements of only visible light region (800 nm–380 nm).

2.2.3 Elemental analysis of samples

Inductively coupled plasma-optical emission spectroscopy

Samples that were acquired from the greenhouse trial were analyzed with inductively couple plasma-optical emission spectroscopy for their elemental content [124]. Samples were prepared as follows:

1. 5 average looking seeds were selected from each sample pool to make an amount between 0.2 g and 0.3 g. They were weighed and. DWF and blank were among samples for control. These samples were transfered to containers.

2. 5 ml of 65% nitric acid was added to each container and incubated for 10 minutes.

3. 2 ml of 30% hydrogen peroxide was added to each container.

4. The containers were locked and put into the microwave oven and incubated for half an hour at 1200 W.

5. After samples were allowed to cool, adequate ddH_2O was added to the containers to make a final volume of 20 ml.

Processed samples were analyzed with ICP-OES for zinc as well as other elements (Ca, K, P, S, Mg, Fe, Mn, Cu, Al, Na). Standarts were also analyzed for calibration of the device.

Dumas combustion and GPC estimation

Dumas combustion was carried out automatically by LECO TruSpec CN for the determination of nitrogen and carbon percentages in the seed samples [125]. Seeds were selected of each sample to make approximately 0.2 g for the analysis. These seeds, along with reference pulverized peach leaf, were wrapped in tin foils and placed in the equipment.

To approximate the grain protein content of the seeds, nitrogen percentages were multiplied by a factor of 5.83 [126].

2.2.4 Protein extraction

Protein extractions were done on whole seed samples, as well as endosperm and embryo sections. Endosperm and embryo sections were seperated under a dissecting microscope. After the embryo was removed from the seed, scutellum and aluerone layer was removed from endosperm by sanding. Because of high protein content of embryos, 2/3 of required meal in Osborne fractionation and half of required meal in extraction for SDS analysis is used.

Osborne fractionation

A modified Osborne fractionation protocol based on Fido et al. [127] was used. This method makes use of different solvents, both organic and aqueous, to extract seed proteins. Seeds were ground before extraction. One gram of meal was incubated in 10 mL of respective solvent while stirred constantly. Each extraction step was carried for 1 hour at 20°C if not otherwise stated and steps were repeated once. At the end of each extraction step, the mixture was

centrifuged for 20 minutes at 10000 g. Supernatants of the same extraction step were combined and saved at 4°C for analysis. Proceeding steps used the pellet from the previous extraction. The procedure is as follows:

1. Extract lipids with water saturated 1-butanol.

2. Resuspend the pellet in 0.5 M NaCl to extract salt soluble albumins, globulins and non-protein components. Include 1 mM PMSF. Work at 4°C.

3. Resuspend the pellet in ddH_2O to remove residual NaCl. Vortex shortly, centrifuge and combine this supernatant with the supernatant from step 2.

4. Resuspend the pellet in 50% (v/v) 1-propanol to extract monomeric prolamins and alcohol soluble stabilized proteins. This step may be omitted.

5. Resuspend the pellet in 50% (v/v) 1-propanol containing 1% (v/v) 2-mercaptoethanol and 1% (v/v) acetic acid to extract reduced subunits of alcohol insoluble disulfide bonded polymers.

6. Resuspend the pellet in 0.05 M borate buffer pH 10 containing 1% (v/v) 2-mercaptoethanol and 1% (w/v) SDS to extract glutelins.

Supernatants were either used directly or dialysed against ddH_2O for 48 hours at 4°C with several changes of dialysis buffer. Samples were lyophilized after dialysis. Originally, the following was suggested [127] for the supernatants, but was not followed:

Supernatant 1 (SUP1) (from water saturated 1-butanol extraction) Discard as this fraction only contains lipids.

Supernatant 2–3 (SUP2–3) (combination of the supernatants acquired during 0.5 M NaCl extraction and residual NaCl removing step) Dialyse against several changes of ddH_2O for 48 hours at 4°C. Centrifugation after dialysis removes globulins and lyophilization allows soluble albumins to be recovered.

Supernatant 4a (SUP4a) (from 50% 1-propanol extraction) Prolamins may be recovered upon precipitation at 4°C overnight by dialysis against ddH_2O or by addition of two volumes of 1.5 M NaCl solution.

Supernatant 4b (SUP4b) (from 50% 1-propanol with reducing agents extraction) Procedure is the same as for supernatant 4a.

Supernatant 5 (SUP5) (from 0.05 M borate buffer extraction) Glutelins are recovered after dialysis against ddH_2O and lyophilization. SDS may be removed using standart procedures.

A modification by Singh *et al.* [128] to Osborne fractionation procedure was followed to be able to extract gliadins and glutenins. This method was modified slightly and several chemicals were replaced for practical reasons:

1. For 20 mg of crushed kernel, extract gliadins in 1 ml of 50% (v/v) 1-propanol at 65°C with intermittent vortexing for 30 minutes. Centrifuge for 5 minutes and save the supernatant for further analysis.

2. Repeat above step, discard the supernatant. Wash the pellet with 0.5 ml of 50% (v/v) 1-propanol. Centrifuge for 5 minutes and discard the supernatant. The pellet is now free of gliadins.

3. Extract glutenins in 0.1 ml of 50% (v/v) 1-propanol with 0.08 M Tris-HCl pH 8.0 containing 1% (w/v) DTT at 65°C for 30 minutes. Do not

vortex except for the beginning of this step. Centrifuge for 5 minutes. Save the supernatant containing reduced glutenins, discard the pellet.

Native extraction

It is also favorable to extract proteins in their native state. For the purpose, the ground seeds were incubated in 62.5 mM Tris-HCl pH 6.8 buffer containing 1 mM PMSF to inhibit protease activity and 0.1 mM $ZnSO_4$ for the stability of metal binding proteins. Extraction was carried at 4°C for 20 hours under agitation.

Extraction for SDS-PAGE analysis

For the bulk extraction of seed proteins, the method based on Fido et al. [127] was used. Steps were as follows:

1. Grind the seeds in a mortar with 25 μl of SDS extraction buffer per milligram of meal.
2. Transfer the ground seed and buffer mixture to eppendorf tubes and leave at least 2 hours while vortexing.
3. Boil at 95°C for 5 minutes.
4. Allow to cool, then spin in a microfuge.
5. Load the supernatant directly to SDS-PA gels and separate the proteins.

2.2.5 S-carboxymethylation of cysteine

For some applications, after reducing with β-mercaptoethanol or DTT, proteins of some extracts are incubated in buffers containing 100 mM IAA at

65°C for 15 minutes. This procedure alkylated cysteine residues of the proteins irreversibly [129].

2.2.6 Bradford assay and protein concentration approximation

For the determination of protein concentrations method of Bradford [130] is used. Reagents were purchased from Bio-Rad and Sigma-Aldrich. For the standart curve, following concentrations (in mg/ml) of BSA solutions were prepared in ddH_2O: 0.050, 0.075, 0.100, 0.150, 0.200, 0.300, 0.400, 0.500, 0.750, 1.000, 1.250, 1.500. Assays were done on 96 well plates. Bio-Rad reagent concentrate was diluted with 5 parts water and 200 μl of diluted reagent was added to 10 μl of protein solutions. Sigma-Aldrich reagent was used as purchased and 250 μl of reagent was added to 5 μl protein solutions. Following the addition of reagents, absorbances were measured at 595 nm after a minimum of 5 minutes.

For the cases, where method of Bradford could not be applied due to the use of already colored buffers, spectrophotometry was used to estimate the protein content. Absolute concentrations were not possible to find, since extinction coefficients of extracts were not known. Absorbances at 230 nm and 260 nm were noted and concentrations of the samples were determined relative to each other.

2.2.7 Electrophoresis

Protein samples and extracts were run on polyacrylamide gels as described [131]. SDS gels and native gels were prepared according to the recipes at Tables 2.3 and 2.4. After preparation of the mixture, the gels were allowed to polymerize at 4°C for a minimum of 12 hours. Running buffers were used at room temperature and gels were not equilibrated with the running buffer before

the run. Proteins that were extracted for SDS-PAGE analysis were loaded directly to the gel, whereas Osborne fractions were mixed with Laemmli buffer and incubated at 95°C for 5 minutes. Samples containing alcohol in extraction solutions like SUP4, were incubated at 70°C for an additional 5 minutes with caps on, in order to evaporate the alcohol. Alcohol prevented samples to fill in the wells of PA gels. Samples were analyzed on native gels with native sample buffer without prior boiling.

Depending on the gel thickness and well sizes, 20–30 mg of protein was loaded. Optimum amount to load to the gels were chosen on a trial and error basis, where it was not possible to calculate absolute protein amount. To emphasize the differences between protein profiles, amount to load to the gels were normalized after estimating the protein concentration of the samples by spectrophotometry and Bradford assays. Assessing yield of different extraction methods was done by loading same volumes of extract.

Electrophoresis was carried out at 20 mA constant current for stacking of proteins and at 30 mA constant current for resolution in PAGE running buffers. During the run, if the buffer temperature or resistance increased too high, it was replaced. Electrophoresis was carried out until the leading dye mark reached the bottom of the gel.

2.2.8 Gel staining

After the electrophoresis, the gels were either stained in coomassie staining solution or were directly used for blotting. Duration of staining was approximately 2 hours. Stained gels were destained with boiling ddH_2O. After documentation of gels, they were either air dried between two sheets of cellophane or vacuum dried for preservation.

A metal staining procedure is also experimented. Instead of staining

Stacking Gel: Material	Final Concentration
ddH_2O	
1 M Tris-HCl pH 6.8	50 mM
20% SDS	0.1%
30% Acryl-0.8% Biacryl	5%
10% APS	0.075%
100% TEMED	0.05%

Resolving Gel: Material	Final Concentration
ddH_2O	
3 M Tris-HCl pH 8.9	375 mM
20% SDS	0.1%
30% Acryl-0.8% Biacryl	12% or 15%
10% APS	0.075%
100% TEMED	0.05%

Table 2.3: Recipe of the SDS polyacrylamide gels.

native gels with Coomassie Brilliant Blue R-250, solutions with heavy metal indicators are prepared. Gels are incubated in these solutions of DTZ and DEDC. Solutions experimented were 0.01% DTZ in 100% ethanol or 0.001% DTZ in 40% methanol with 10% acetone or 100 mM DEDC in ddH_2O.

2.2.9 Blotting

Proteins were transfered from polyacrylamide gels to Hybond-P PVDF membranes [132] using the wet transfer technique [133] for detection of zinc binding. After separation of proteins, before the transfer, the SDS-PA gels were incubated in reducing buffer for blotting for 1 hour at 37°C. For transfer from SDS-PA gels, SDS transfer buffer, for transfer from native gels, native transfer buffer was used. The membranes were activated by 100% methanol prior to transfer.

Stacking Gel: Material	Final Concentration
ddH_2O	up to final volume
1 M Tris-HCl pH 6.8	50 mM
30% Acryl-0.8% Biacryl	5%
10% APS	0.075%
100% TEMED	0.05%

Resolving Gel: Material	Final Concentration
ddH_2O	up to final volume
3 M Tris-HCl pH 8.9	375 mM
30% Acryl-0.8% Biacryl	8%
10% APS	0.075%
100% TEMED	0.05%

Table 2.4: Recipe of the native polyacrylamide gels.

Electrophoretic transfer was done at 200 mA constant current for 90 minutes at 4°C. After the transfer, the efficiency was assesed by staining the gel with coomassie staining solution. Transfers of low efficiency were discarded.

2.2.10 Detection of zinc binding

Procedure of Mazen et al. [119] and Schiff et al. [120] was modified to replace radioactive ^{65}Zn(II) with non-radioactive Zn(II). Procedure is as follows:

1. Keep the membrane on the rotator during the procedure.
2. For the renaturation of the proteins, soak the PVDF membrane with the transfered proteins in renaturation buffer containing 0.1% Triton X-100 for one hour at 4°C. Triton X-100 will remove SDS.
3. Wash the membrane in zinc incubation buffer for 15 minutes at 4°C.

4. Incubate the membrane in zinc incubation buffer containing 100μM $ZnCl_2$ for 15 minutes at 20°C.

5. Wash the membrane 3 times for 10 minutes each time in zinc incubation buffer at 20°C.

6. Spray metal indicator solutions onto the membrane on a clean zinc-free surface.

If staining was poor, sprayed membrane was rapidly dipped in 100% methanol to get brighter and more intense staining. Digital photographs of the processed membranes were taken and the images were digitally enhanced with GIMP 2.2.12 software.

To test the validity of this method, various control runs has been performed. These included replacement of zinc with cadmium, nickel, 5 mM EDTA, water; applying different concentrations of zinc; loading different amounts of protein; and including reducing agents (5 mM TCEP-HCl) during procedure. The membranes were also destained with 100% methanol of metal indicators to be later stained by Ponceau S for protein content verification.

Sometimes, it was desired to observe the bands on the gels before blotting, therefore they were prestained by coomassie staining solution. After the electrophoretic transfer, the dye also passed onto the membrane. In these cases, the membrane was destained of coomassie with 100% methanol before proceeding with the protocol.

2.2.11 Seed sections and microscopy

Longitutional seed sections were stained for zinc with DTZ or PAN solutions to see the localization of the metal. The method was a modification to the

method of Ozturk et al. [115]. Selected seeds with high zinc content were left to start germination at room temperature between water soaked tissue papers and in dark. Germination was arrested at various times by removing the seed from the set-up and flash freezing with liquid nitrogen. Seeds were collected at these times: 0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 90 min, 2 hr, 3 hr, 4 hr and 5 hr. These seeds were later broken in halves through longitudinal sections in a mortar with a pestle. Later, staining was applied and the results were recorded using a zoom stereo microscope and a digital camera.

Same procedure was repeated with 1% ninhydrin in 100% ethanol solution, this time to stain the proteins and see the localization of the proteins. Ninhydrin is a pale yellow solution that turns purple upon interaction with amines of polypeptides. Seed sections were incubated in the ninhydrin solution for increasing intervals of time at 70°C.

2.2.12 Statistical methods

Measurements representing each group of plants grown in the greenhouse are arithmetic averages of five paralles in the group. Error value is the standart deviation of the measurements from five parallels.

Chapter 3

Results

3.1 Effect of varying nitrogen and zinc concentrations on *Triticum durum* growth and seed properties

3.1.1 Dry matter production

It is seen that increasing nitrogen and zinc nutrition had a positive effect on the dry matter and seed production (Table 3.1, Figure 3.1). However, nitrogen concentrations higher than 225 ppm and zinc concentrations higher than 8 ppm, had no significant positive effect, even in some cases, had a negative one. Foliar applications do not seem to have any observable outcome.

3.1.2 Element analyses of seeds

Analyzed results for element analyses of zinc, phosphorus, nitrogen and GPC are summerized at Table 3.2 and Figure 3.2. Zinc accumulation in the seeds were proportional to the zinc application during growth. As the applied zinc concentration was increased, the accumulation in the seeds was also increased. It is noteworthy that an increase in the nitrogen application from 75 ppm to 225 ppm, increased zinc accumulation by approximately 30%. Higher concentrations of nitrogen application did not show significant differ-

Total Dry Weight (g.)	Nitrogen Concentration (ppm) 75	225	675	675+foliar
0.5 ppm Zn	2.14 ± 0.27	2.42 ± 0.33	2.46 ± 0.43	2.31 ± 0.38
8 ppm Zn	1.72 ± 0.28	2.94 ± 0.39	2.36 ± 0.11	2.48 ± 0.28
24 ppm Zn	1.72 ± 0.24	3.05 ± 0.53	2.22 ± 0.26	2.23 ± 0.22
Seed Weight (g.)	75	225	675	675+foliar
0.5 ppm Zn	1.34 ± 0.13	1.55 ± 0.12	2.07 ± 0.53	1.91 ± 0.16
8 ppm Zn	1.36 ± 0.27	1.74 ± 0.10	1.98 ± 0.22	1.87 ± 0.20
24 ppm Zn	1.39 ± 0.15	1.56 ± 0.32	1.63 ± 0.26	1.62 ± 0.31

Table 3.1: Total dry weight and seed production of plants grown at the greenhouse. Values are per plant average weights and standart deviations of five independent replications. Tables are total dry weights and produced seed weights averages. Labels correspond to the growth conditions as described in Section 2.2.1. Data from individual samples can be found in Appendix A.1.

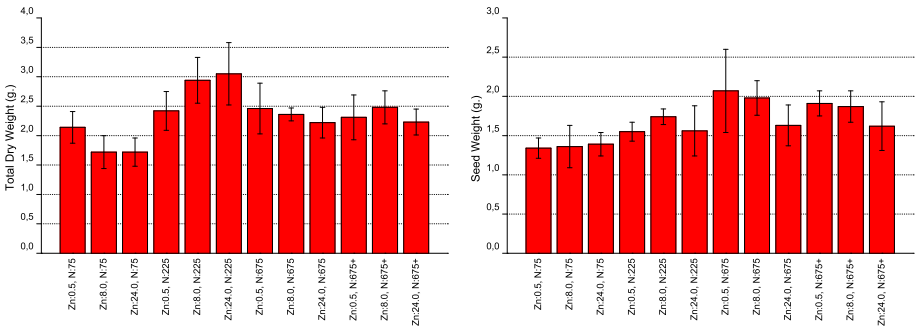

Figure 3.1: Total dry weight and seed production of plants grown at the greenhouse. Growth conditions, shown below the bars, are quantities in ppm. Quantities with a (+) symbol indicate foliar application of that nutrition.

ences on the zinc content.

Phosphorus accumulation was independent of zinc application, however, nitrogen applications of 625 ppm and higher resulted the accumulated phosphorus concentration to drop by approximately 15%.

The nitrogen content in the seeds, thus grain protein content increased with the increase in the nitrogen application. However, as in zinc accumulation, concentrations higher than 225 ppm nitrogen did not show important variations. At nitrogen applications higher than 225 ppm, increasing zinc application affected nitrogen accumulation positively, but at 75 ppm of nitrogen application, zinc application had a negative effect.

3.2 DTZ and PAN properties

Interactions of metal indicators DTZ and PAN with various metals (Zn, Cd, Ni, Mn, Cu, Hg, Pb) were investigated both visually and with spectrophotometry. Color of pure DTZ and PAN showed significant changes upon binding with metals. Figure 3.3 shows these changes visually. Neither DTZ nor PAN interacted with manganese. Although DTZ interacted with rest of the heavy metal solutions, PAN did not interact with cadmium or mercury. The color acquired by their interaction with zinc is similar. Concentration of the indicators determines the intensity of the color of solutions, whereas concentration of the metal determines how much colors will shift.

Spectrum of different colors acquired by interactions with excess concentions of metals are shown on Figure 3.4. Different metals gave peaks at different wavelengths with different absorbances.

Visually, an increase of the concentration of the indicators was described as an increase in the intensity or saturation of the color. There is, actually, an increase in the absorbances of the solutions, proportional to the increase

	Nitrogen Concentration (ppm)			
Zn (ppm)	75	225	675	675+foliar
0.5 ppm Zn	18.0 ± 1.1	16.3 ± 2.9	15.7 ± 2.8	16.8 ± 1.7
8 ppm Zn	62.0 ± 2.5	79.2 ± 5.6	74.1 ± 6.3	76.1 ± 3.6
24 ppm Zn	77.0 ± 9.8	110.2 ± 10.7	94.5 ± 7.3	100.8 ± 15.4
P (ppm)	75	225	675	675+foliar
0.5 ppm Zn	5005.2 ± 111.7	5068.7 ± 238.7	4411.8 ± 334.3	4222.6 ± 184.7
8 ppm Zn	5035.8 ± 127.1	4981.6 ± 199.0	4248.6 ± 223.8	4322.8 ± 299.2
24 ppm Zn	4972.3 ± 233.3	5269.5 ± 129.4	4347.9 ± 407.3	4588.6 ± 377.7
N (%)	75	225	675	675+foliar
0.5 ppm Zn	2.67 ± 0.09	3.04 ± 0.07	3.11 ± 0.19	3.11 ± 0.02
8 ppm Zn	2.52 ± 0.19	3.26 ± 0.05	3.15 ± 0.08	3.17 ± 0.05
24 ppm Zn	2.49 ± 0.20	3.33 ± 0.15	3.24 ± 0.07	3.31 ± 0.20
GPC (%)	75	225	675	675+foliar
0.5 ppm Zn	15.59 ± 0.52	17.72 ± 0.39	18.16 ± 1.10	18.14 ± 0.13
8 ppm Zn	14.72 ± 1.11	19.02 ± 0.27	18.35 ± 0.45	18.48 ± 0.30
24 ppm Zn	14.52 ± 1.19	19.42 ± 0.88	18.87 ± 0.41	19.30 ± 1.17

Table 3.2: Element accumulation in the seeds of samples acquired from greenhouse experiment. Values are averages and standart deviations of five independent replications. Labels correspond to the growth conditions as described in Section 2.2.1. Data from individual samples can be found in Appendix A.2 and A.3.

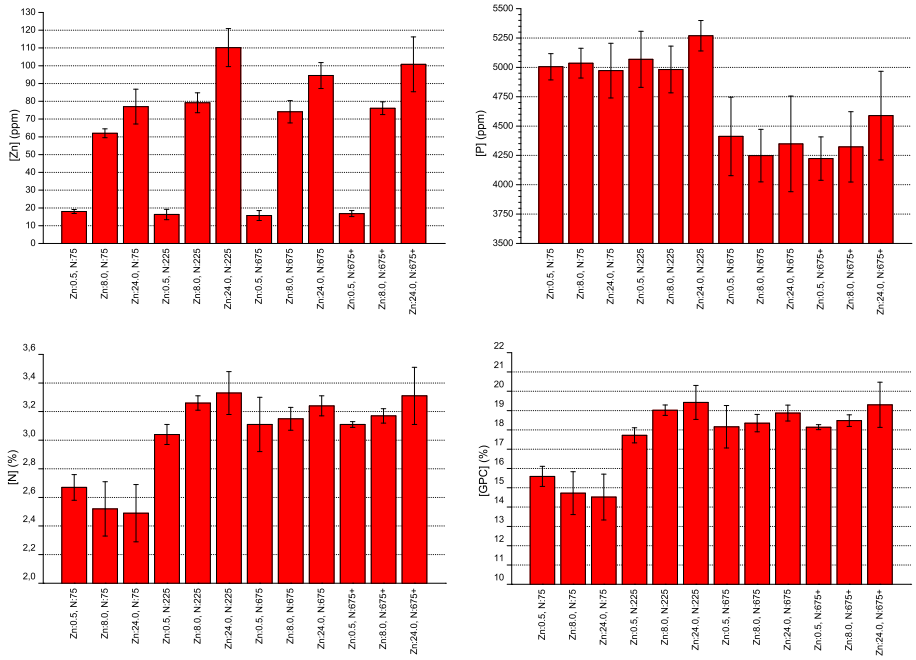

Figure 3.2: Element accumulation in the seeds of samples acquired from greenhouse experiment. Grain protein content was calculated from nitrogen percentages. Growth conditions, shown below the bars, are quantities in ppm. Quantities with a (+) symbol indicate foliar application of that nutrition.

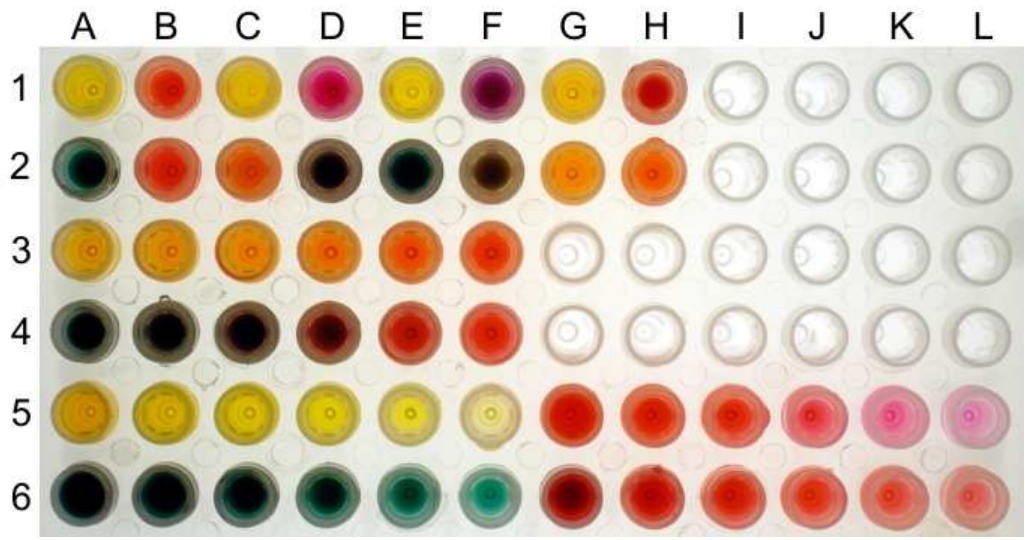

Figure 3.3: PAN and DTZ interacting with indicated metal solutions at indicated concentrations. **1A:** 50 μM PAN. **1B-1H:** 50 μM PAN with 5 mM $ZnCl_2$, $CdSO_4$, $NiCl_2$, $MnSO_4$, $CuSO_4$, $HgCl_2$, $Pb(NO_3)_2$. **2A:** 50 μM DTZ. **2B-2H:** 50 μM DTZ with 5 mM $ZnCl_2$, $CdSO_4$, $NiCl_2$, $MnSO_4$, $CuSO_4$, $HgCl_2$, $Pb(NO_3)_2$. **3A-3F:** 50 μM PAN with 100 μM, 200 μM, 400 μM, 800 μM, 1.6 mM and 3.2 mM $ZnCl_2$. **4A-4F:** 50 μM DTZ with 100 μM, 200 μM, 400 μM, 800 μM, 1.6 mM and 3.2 mM $ZnCl_2$. **5A-5F:** 250 μM, 125 μM, 67.5 μM, 33.5 μM, 16.8 μM and 8.4 μM PAN. **5G-5L:** 250 μM, 125 μM, 67.5 μM, 33.5 μM, 16.8 μM and 8.4 μM PAN with 5 mM $ZnCl_2$. **6A-6F:** 250 μM, 125 μM, 67.5 μM, 33.5 μM, 16.8 μM and 8.4 μM DTZ. **6G-6L:** 250 μM, 125 μM, 67.5 μM, 33.5 μM, 16.8 μM and 8.4 μM DTZ with 5 mM $ZnCl_2$.

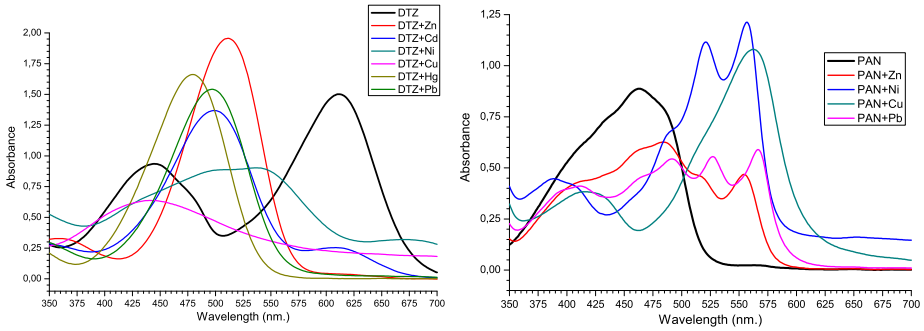

Figure 3.4: Spectrum of DTZ, PAN and their metal complexes. Metals interacting are Zn, Cd, Ni, Cu, Hg and Pb for DTZ, and Zn, Ni, Cu, and Pb for PAN. 50 μM DTZ and PAN in acetone is used. Metal solutions are 5 mM.

of the concentrations (Figure 3.5).

Shifting of colors due to the addition of metal solutions are seen as a loss of the characteristic peaks of indicators, whilst emergence of a new peak that is specific to the metal (Figure 3.6).

3.3 Protein extraction from *T. durum* seeds

3.3.1 Analysis of total and fractionated protein extracts

Prior to the analysis of seeds from the greenhouse experiment, several reference genotypes were chosen to optimize protein extraction methods. Genotype Bezostaja was used for most of the initial experimentation. TAM107, Kharkov and Scout66 genotypes were chosen for comparison with previously identified protein bands in Budak et al. [134]. Three extraction methods, as noted in Section 2.2.4, were used; Osborne fractionation, extraction for SDS-PAGE analysis and native extraction.

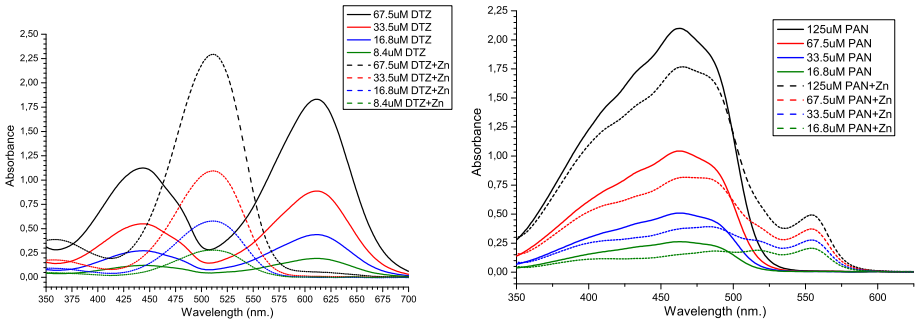

Figure 3.5: Spectrum of different concentrations of DTZ and PAN with and without 5 mM of $ZnCl_2$. DTZ concentrations are 67.5 μM, 33.5 μM, 16.8 μM and 8.4 μM. PAN concentrations are 125 μM, 67.5 μM, 33.5 μM and 16.8 μM.

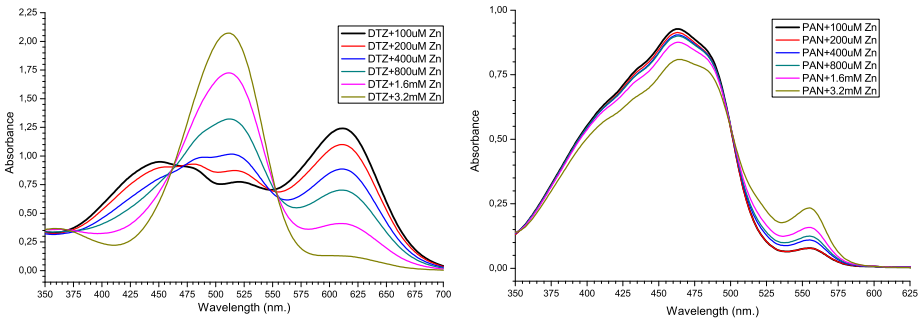

Figure 3.6: 50 μM DTZ and PAN with increasing concentrations of zinc. $ZnCl_2$ concentrations are 100 μM, 200 μM, 400 μM, 800 μM, 1.6 mM and 3.2 mM.

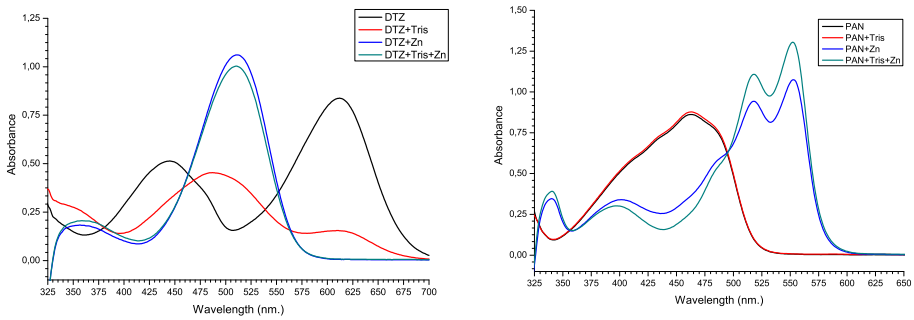

Figure 3.7: Spectrum of interactions of 50 μM DTZ and PAN with 5 mM Tris-HCl pH 8.9 and 5 mM $ZnCl_2$.

Protein yields of Osborne fractionation and native extraction were determined by Bradford assays and spectrophotometry and the yield of total extraction was determined by spectrophotometry alone. The concentrations values were then used to normalize the amount for loading for electrophoresis. Amount to be loaded were normalized to be able to make better comparisons between samples. Gels were stained with Coomassie Brilliant Blue R-250, which initially resulted in the low molecular weight proteins being not clearly visible when compared to the mid range proteins, however with improved quality gels and use of fresh staining solutions visible bands were acquired.

Preliminary results and comparison of these methods can be seen on Figures 3.8 and 3.9. Samples of total extraction are loaded in lanes 2–4 of Figure 3.8 at different amounts for better visualization. Of all methods, best yield per gram of ground seed is acquired by total extraction. Still, it was possible to adjust the concentration of the Osborne fractions after lyophilization. As expected, proteins extracted for SDS-PAGE analysis appear as overlay of

different Osborne fractions. Native extraction, when compared with total extraction, does not include all the proteins, but rather gives a similar protein span to the SUP2–3 fraction of Osborne procedure.

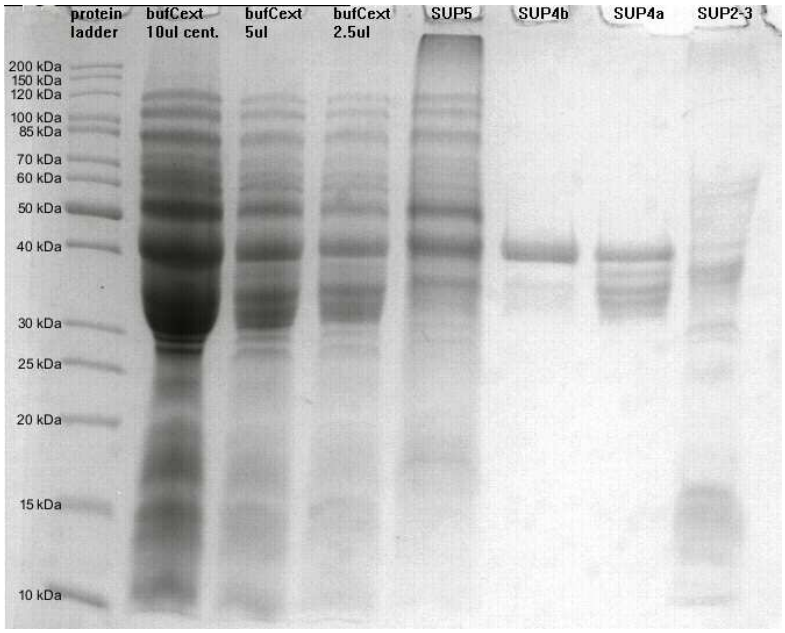

Figure 3.8: SDS-PAGE analysis of seed proteins extracted by total extraction and Osborne fractionation. Extracts are from T. durum, genotype Bezostaja. Gel is 12% SDS-PA.
Lane 1: PageRuler protein ladder. Molecular weights are given on the left.
Lanes 2–4: 10 μl, 5 μl and 2.5 μl loading of total extracts.
Lanes 5–8: Osborne fractions. SUP5, SUP4b, SUP4a and SUP2–3 (See Section 2.2.4 on Page 33).

3.3.2 Analysis of total and fractionated protein extracts from *T. durum* grown under varying nitrogen and zinc concentrations

Four samples were chosen from the greenhouse experiments as models, according to growth conditions. Seeds were selected to represent those grown under low nitrogen concentration with low zinc concentration, low nitrogen

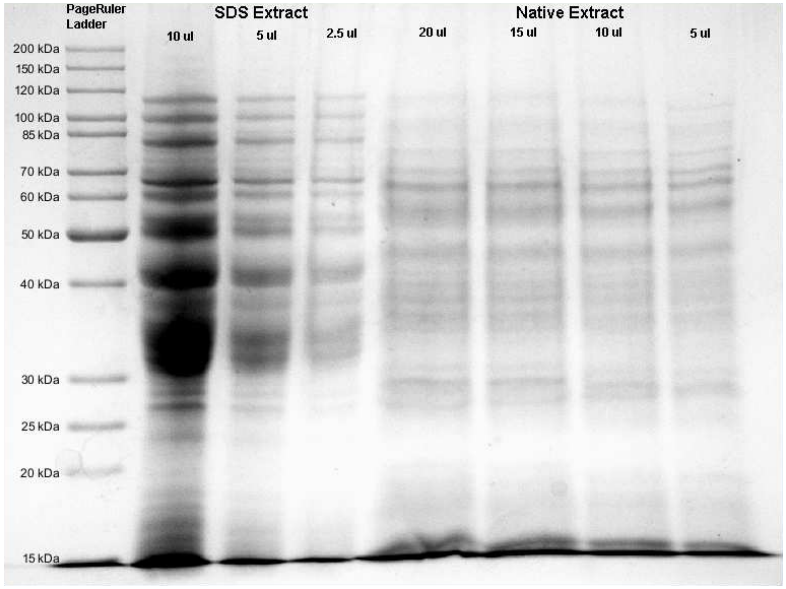

Figure 3.9: SDS-PAGE analysis of seed proteins extracted by total extraction and native buffers. Extracts are from T. durum, genotype Bezostaja. Gel is 10% SDS-PA.
Lane 1: PageRuler protein ladder. Molecular weights are given on the left.
Lanes 2–4: 10 μl, 5 μl and 2.5 μl loading of total extracts.
Lanes 5–8: 20 μl, 15 μl, 10 μl and 5 μl loading of native extracts.

concentration with high zinc concentration, high nitrogen concentration with low zinc concentration and high nitrogen concentration with high zinc concentration. Accordingly, it is expected that samples grown under low nitrogen concentration to have low protein content and samples grown under low zinc concentration to have low zinc content. Similarly, high accumulations were expected for samples grown under high concentrations of nitrogen and zinc.

All of the extraction methods have been applied to these seeds and extracts were compared on SDS-PA and native gels. As SUP5 fraction of the Osborne procedure was very similar to total extracts in terms of both protein profiles (Figure 3.8) and the chemistry of solvents, the Osborne procedure was followed only until the end of step 5 for practical reasons and time con-

straints. SUP4 was extracted in one step, therefore SUP4a and SUP4b does not exist for these samples. Figure 3.10 shows the first three fractions. SUP1 was loaded as a verification that proteins were not extracted at this step. On this application of Osborne procedure, the extracts were not dialysed or lyophilized, but used directly to avoid losses due to precipitation during dialysis. This resulted in better yields as well as more accurate protein profiles.

As can be seen in Figure 3.10, the fractionated components from seeds obtained under different environmental conditions appear very similar.

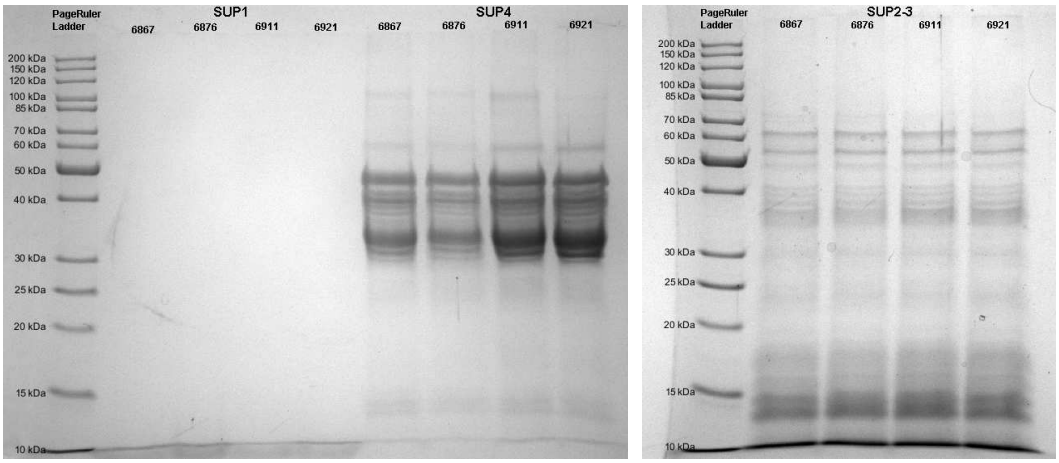

Figure 3.10: SDS-PAGE analysis of Osborne fractions of greenhouse samples. Gels are 12% SDS-PA.
Lanes 1, 9: PageRuler protein ladder. Molecular weights are given on the left.
Lanes 2–5: SUP1 of greenhouse samples 6867, 6876, 6911, 6921.
Lanes 6–9: SUP4 of greenhouse samples 6867, 6876, 6911, 6921.
Lanes 10–13: SUP2–3 of samples 6867, 6876, 6911, 6921.

Comparison of total protein extractions by SDS of greenhouse samples with each other and with reference samples for identification of the protein bands is shown in Figure 3.11.

Protein profiles of different regions of the seeds, representing low content and high content, were also investigated. Total extraction and Osborne frac-

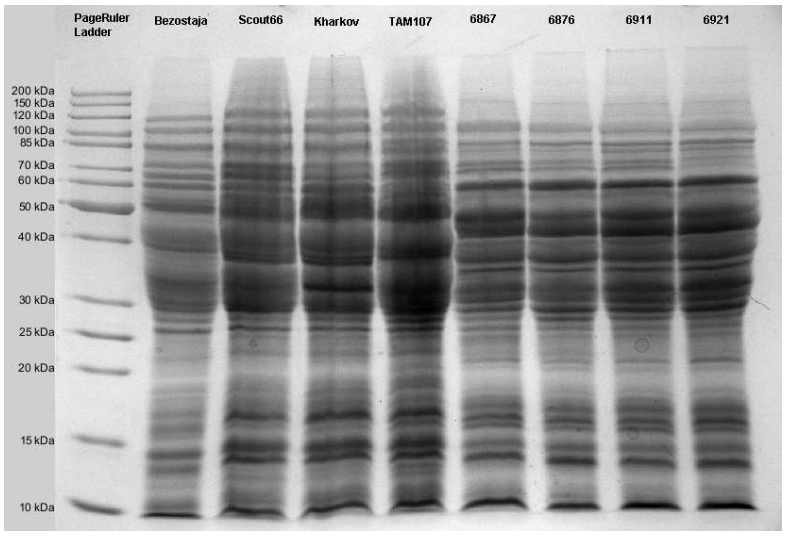

Figure 3.11: SDS-PAGE analysis of total protein extracts from reference samples and greenhouse samples. Gel is 12% SDS-PA.
Lane 1: PageRuler protein ladder. Molecular weights are given on the left.
Lane 2: Extraction from Bezostaja. Lane 3: Extraction from Scout66.
Lane 4: Extraction from Kharkov. Lane 5: Extraction from TAM107.
Lanes 6–9: Extraction from samples 6867, 6876, 6911, 6921.

tionation were carried out for the embryo and the endosperm. Although care was taken in dissections, extracts may have cross-contaminations. Differences in the protein concentrations (Table 3.3) and differences in protein profiles (Figure 3.12) between various regions of the seed are presented. As can be seen in the table, second extraction steps have 3-4 fold less protein and for this reason these supernatants were not combined to avoid the dilution of proteins in the first extract. Notable and expected differences in the protein concentration of low and high content samples were not seen, however, differences between extracts of embryo and endosperm were observed. Protein concentrations in the mild saline extracts are much higher in embryos, but this is reversed in propanol extracts. When comparing the total extracts, it can be seen that embryo extractions have higher protein concentrations

than endosperm extracts. Extraction and load amounts were normalized for protein amount to emphasize the differences between profiles.

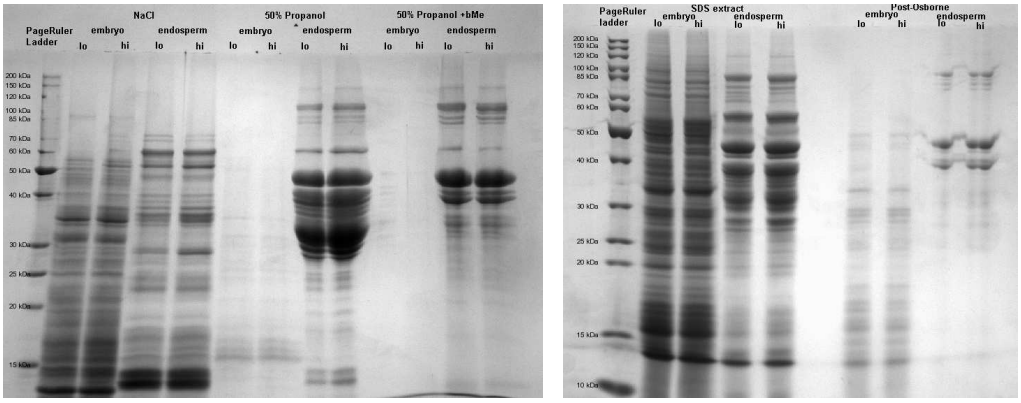

Figure 3.12: SDS-PAGE analysis of Osborne fractions (left gel) and total protein extracts (right gel) of parts of the seed. Residual protein content of samples after Osborne fractionation is also shown (right gel). "lo" denotes samples of low nitrogen, low zinc content (blend of seeds 6864, 6866) and "hi" denotes samples of high nitrogen, high zinc content (blend of seeds 6890, 6891, 6920, 6922, 6889). Samples are loaded after normalizing their concentration. In cases where protein content of the extract was very low, such as in propanol solution extracts of high and low embryo samples, samples are loaded at the maximum volume the wells can take. Gels are 12% SDS-PA.
Left:
Lane 1: PageRuler protein ladder. Molecular weights are given on the left.
Lanes 2–5: 0.5 M NaCl extracts (SUP2–3) of embryo and endosperm.
Lanes 6–9: 50% propanol extracts (SUP4a) of embryo and endosperm.
Lanes 10–13: 50% propanol with reducing agent extracts (SUP4b) of embryo and endosperm.
Right:
Lane 1: PageRuler protein ladder. Molecular weights are given on the left.
Lanes 2–5: Total protein extraction of embryo and endosperm.
Lanes 7–10: Total protein extracts of the final pellet of Osborne fractionation.

To see the effect of Singh modification to the Osborne procedure, gliadins and glutenins were extracted from embryo and endosperm sections (Figure 3.13). Gliadin extracts corresponded to SUP4a, glutenin extracts corresponded to SUP4b of the Osborne fractions.

			Meal (g)	Vol. (ml)	Dilution Factor	Extract 1^{st} OD_{280} (Abs.)	Extract 1^{st} Norm.	Extract 2^{nd} OD_{280} (Abs.)	Extract 2^{nd} Norm.
	SUP2–3	embr. (lo)	0.0955	1.50	15.71	1.897	29.796	0.454	7.131
		embr. (hi)	0.1015	1.50	14.78	2.007	29.660	0.517	7.640
		endsp. (lo)	0.0995	1.00	10.05	0.261	2.623	0.055	0.553
		endsp. (hi)	0.1043	1.00	9.59	0.209	2.004	0.061	0.585
	SUP4a	embr. (lo)	0.0955	1.50	15.71	0.057	0.895	0.021	0.330
		embr. (hi)	0.1015	1.50	14.78	0.079	1.167	0.033	0.488
		endsp. (lo)	0.0995	1.00	10.05	0.307	3.085	0.019	0.191
		endsp. (hi)	0.1043	1.00	9.59	0.428	4.099	0.051	0.484
	SUP4b	embr. (lo)	0.0955	1.50	15.71	0.004	0.063		
		embr. (hi)	0.1015	1.50	14.78	0.019	0.281		
		endsp. (lo)	0.0995	1.00	10.05	0.122	1.221		
		endsp. (hi)	0.1043	1.00	9.59	0.176	1.683		
Final	**Pellet**	embr. (lo)	0.0955	1.50	15.71	1.354	21.267		
		embr. (hi)	0.1015	1.50	14.78	1.329	19.640		
		endsp. (lo)	0.0995	1.00	10.05	0.188	1.889		
		endsp. (hi)	0.1043	1.00	9.59	0.262	2.507		
Total	**Extract**	embr. (lo)	0.0330	1.50	45.45	1.736	78.909		
		embr. (hi)	0.0330	1.50	45.45	1.963	89.227		
		endsp. (lo)	0.0330	0.75	22.73	1.383	31.432		
		endsp. (hi)	0.0337	0.75	22.26	0.869	19.340		

Table 3.3: Protein concentration of Osborne and total extractions from embryo (embr.) and endosperm (endsp.). Normalized concentrations are indicated as relative values based on 280 nm UV absorbances at 1 mm path length. Normalized values are calculated by multiplying the absorbance with the dilution factor. Dilution factor is the ratio of extraction volume to the initial weight of sample. SUP2–3, SUP4a and SUP4b are Osborne fractions. Total protein extraction was done on the remaining pellet from Osborne fractionation, also, and the measurements are shown in "Final Pellet" rows. Measurements of the extraction for SDS analysis is shown in "Total Extract" rows. "lo" denotes samples of low nitrogen, low zinc content (blend of seeds 6864, 6866) and "hi" denotes samples of high nitrogen, high zinc content (blend of seeds 6890, 6891, 6920, 6922, 6889). Second extractions are repeated extractions before proceeding to the next solvent.

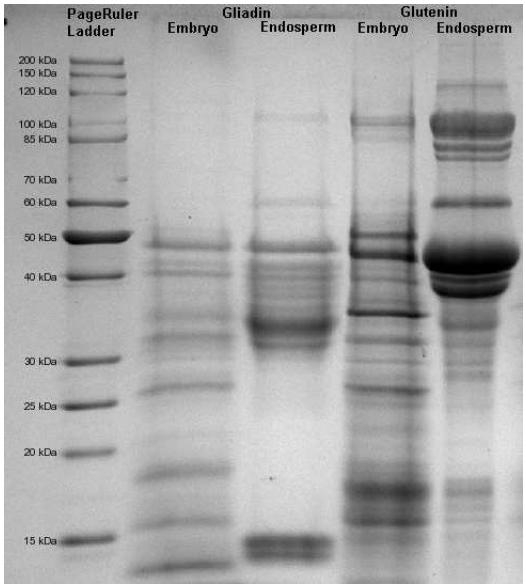

Figure 3.13: Gliadin and glutenin extraction from embryo and endosperm by Singh modifications. Samples are loaded at normalized quantities. Gel is 12% SDS-PA. Lane 1: PageRuler protein ladder. Molecular weights are on the left. Lane 2–3: Gliadin extracts from embryo and endosperm of 6920. Lane 4–5: Glutenin extracts from embryo and endospem of 6920.

Dealing with proteins without disrupting their native structures have definate advantages that shall be discussed elsewhere. Therefore, native extracts were analyzed on native gels, however it was not possible to derive meaningful results (Figure 3.14).

3.4 Detection of Zn-binding with proteins analyzed by PAGE

3.4.1 Analyses after native PAGE

Metal indicator chemicals were used to detect protein bound metals on native PA gels. Solutions of DTZ, PAN and DEDC at various concentrations were prepared and applied to gels using different procedures (see Section 2.2.8). DEDC appeared to be the better canditate of all indicators, because of its solubility in water. However, transparent solution of DEDC only changed color to crimson and precipitated upon binding copper, but only precipitated without any color change upon interaction with other heavy PageRuler protein ladder.metals. Therefore, any attempts to stain gels with aqueous solution

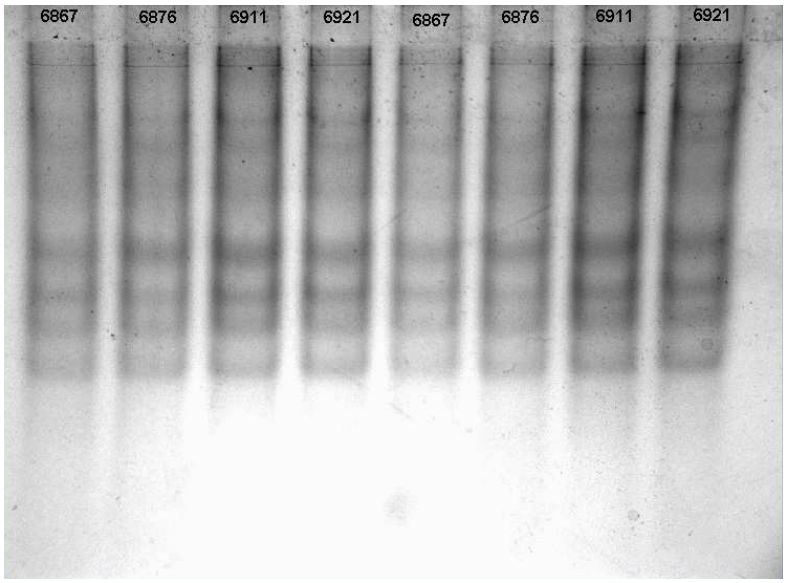

Figure 3.14: Native PAGE analysis of proteins extracted under native conditions. Gel is 8% native PA.
Lanes 1–4: Native extracts of 6867, 6876, 6911 and 6921.
Lanes 5–8: Native extracts of 6867, 6876, 6911 and 6921.

of DEDC was not successful.

Solutions of DTZ in pure organic solvents such as methanol, ethanol and acetone were used, however this resulted a sudden reversible dehydration, thus shrinkage of the gels. Stability of DTZ in the solution was tested by varying the amount of water included, therefore dilutions of the solution in water are very limited and acetone solution can be diluted to have 40% final concentration, which is about the same organic solvent concentration of Coomassie Brilliant Blue R-250 staining solution. It was observed that Tris also interacts with DTZ and the color changes from blue-green to yellow-orange, but this does not affect the interaction with zinc when later introduced.

Application of a bulk solution may dislocate the coordinated metals and

to avoid this DTZ in pure acetone or methanol was sprayed over gels. As can be seen on Figure 3.15, althought the gel changed color, no bands corresponding to seed proteins could be visualized.

Figure 3.15: Native PAGE analysis of total native extracts from T. durum seeds, stained by sprayed DTZ. After electrophoresis, the gel was stained by spraying with DTZ solution. Note the pink blobs at the bottom sides due to handling with latex gloves. Gel is 8% native-PA.

3.4.2 Detection and characterization of zinc-binding proteins after blotting

Using an alternative approach to detection metal bound proteins on native gels, attempts were mande to detect the metal binding proteins after they have been separated by SDS-PAGE and blotted on membranes. As mentioned in Section 2.2.9, total extracts or fractionated proteins were separeted by SDS-PAGE and blotted onto PVDF membranes. Membranes were sub-

sequently incubated in renaturing solutions and metal solutions and were stained for metal.

Figure 3.16 shows zinc detection on membranes containing the Osborne fractions of greenhouse samples. As can be seen from the figure, only SUP4, containing mainly prolamines (see Page 33), shows reddish bands reflecting DTZ-Zn interaction on the membrane. Proteins with molecular weights between 60 kDa and 30 kDa are stained for zinc.

Although DTZ is blue-green in solution, membrane is stained yellow due to Tris buffer-DTZ interaction. As DTZ-Zn complex yields a pink solution, reddish regions on membranes correspond to zinc containing parts. PAN is itself yellow in pure solution and does not interact with Tris buffers. PAN-Zn complex is also a pink solution, therefore both staining procedures gave similar results.

To see if there are differences in the protein profiles that bind zinc, Osborne fractions of embryo and endosperm sections of samples representing high content and low content seeds were blotted and stained for zinc (Figure 3.17). On the extracts of propanol solutions (SUP4a and SUP4b) of endosperm, same bands were stained as in the previous figure.

For the verification and credibility of the method, several control experiments were carried out. Each time, replica gels were stained with Coomassie Brilliant Blue R-250 to identify stained bands. Effect of concentration of zinc in the incubation was tested and as can be seen on Figure 3.18, high concentrations of zinc did not necessarily produce more intense bands, therefore further experiments were conducted with lower concentrations of zinc for specificity. Proteins with molecular weights between 60 kDa and 30 kDa are stained for zinc. This result is consistent with SUP4 from Osborne fractionation.

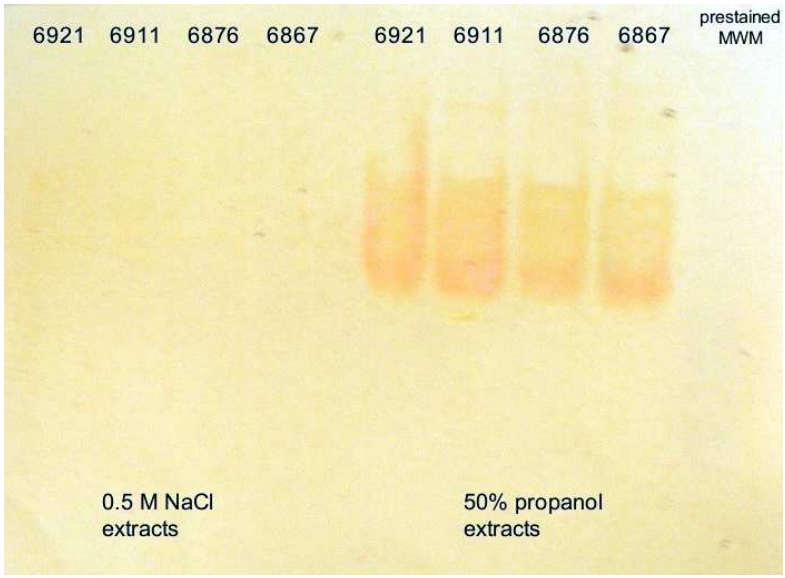

Figure 3.16: Detection of zinc binding to blotted proteins. This membrane is transfered from a gel that is identical to the gels on Figure 3.10. Samples were run on 12% SDS-PA then transfered to PVDF membrane.
Lanes 1–4: SUP2–3 of samples 6921, 6911, 6876 and 6867.
Lanes 5–8: SUP4b of samples 6921, 6911, 6876 and 6867.
Lanes 9: Prestained molecular weight marker.

Association of zinc with membrane bound proteins was investigated by incubating the membrane with 5 mM EDTA instead of zinc. It can be seen in Figure 3.19 that, proteins were not stained in absence of zinc. Here, GST-dMt and BSA was also present in the gels as they are known to bind zinc [135, 136]. This staining shows that, although proteins from endosperm were stained for zinc, there seems to be no visible bands for embryo proteins.

The effect of cadmium incubation can be seen in Figure 3.20. Although no staining is seen in cadmium incubated membrane, when it was rapidly dipped into 100% methanol, same staining pattern was acquired on Samples I and II. Rapid dipping of zinc incubated membrane into methanol yielded bands much intense than those of cadmium incubation.

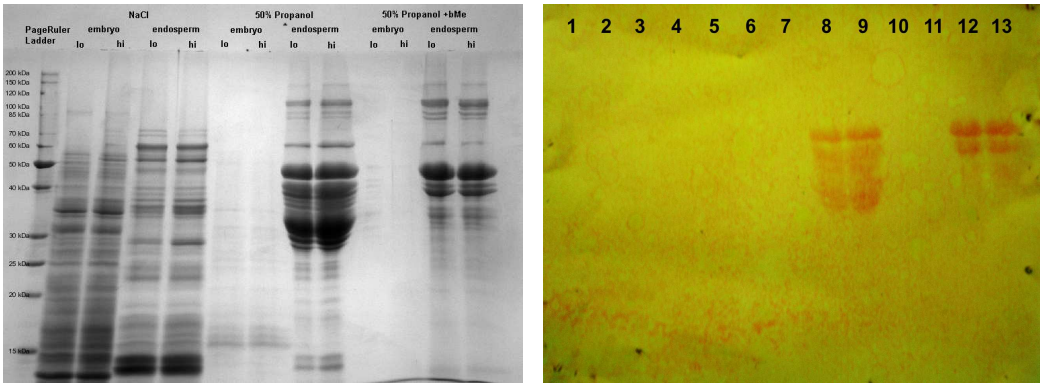

Figure 3.17: Detection of zinc on Osborne fractions of embryo and endosperm tissues. The gel is the same gel as in left part of Figure 3.12.
Lane 1: PageRuler protein ladder. Molecular weights are given on the left.
Lanes 2–5: 0.5 M NaCl extracts (SUP2–3) of embryo and endosperm.
Lanes 6–9: 50% propanol extracts (SUP4a) of embryo and endosperm.
Lanes 10–13: 50% propanol with reducing agent extracts (SUP4b) of embryo and endosperm.

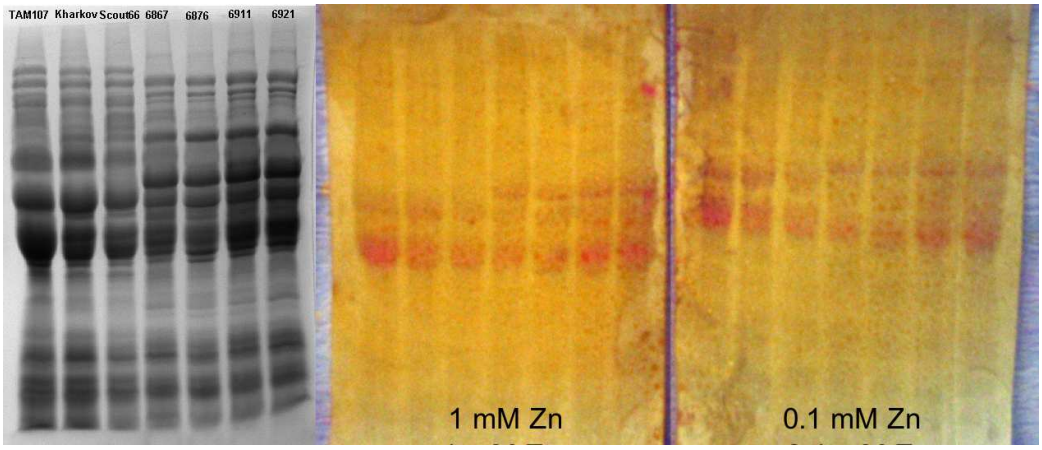

Figure 3.18: Effect of zinc concentration on detection of Zn bound to blotted proteins. The blots are shown with the original gel on the left. Left hand membrane was incubated in 1 M $ZnCl_2$ and right hand membrane was incubated in 0.1 M $ZnCl_2$. Samples were run on 12% SDS-PA. Membrane was stained by sprayed DTZ.
Lanes 1–3: Extracts from TAM107, Kharkov and Scout66.
Lanes 4–7: Extracts from 6867, 6876, 6911 and 6921.

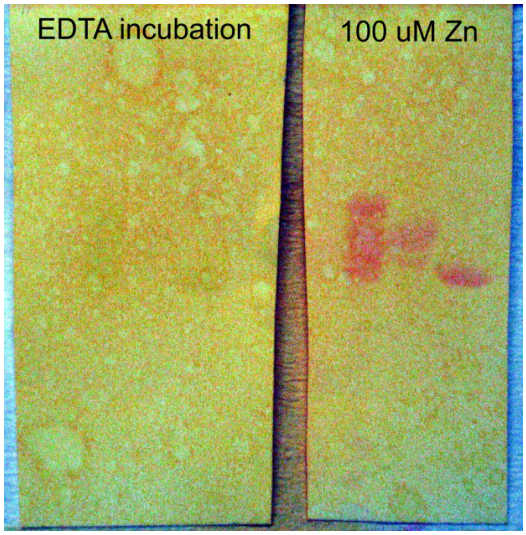

Figure 3.19: Comparison of the effect of EDTA to the effect of zinc incubation in Zn detection. Left membrane was incubated in EDTA, while right membrane was incubated in 0.1 M $ZnCl_2$. No visible bands on EDTA incubation and on embryo extracts.
Lane 1: Embryo extract.
Lane 2: Endosperm Extract.
Lane 3: GST-dMT. Lane 4: BSA

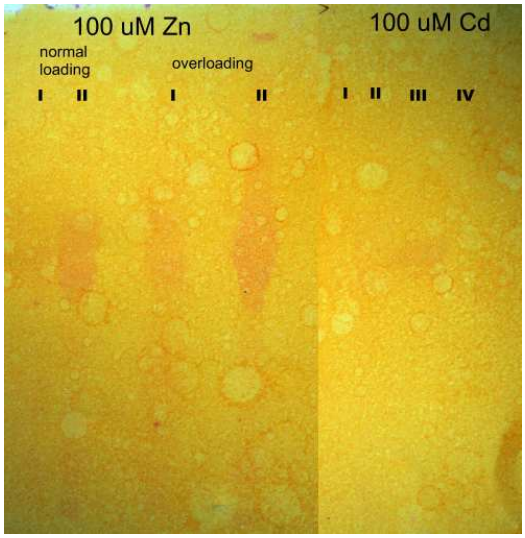

Figure 3.20: Comparison of zinc incubation to cadmium incubation in zinc detection. Left part of the membrane was incubated in 100 μM $ZnCl_2$, while right part of the membrane was incubated in 100 μM $CdCl_2$.
Sample I: total protein extraction from embryo of 6891.
Sample II: total protein extraction from endosperm of 6891.
Sample III: GST-dMT. Sample IV: GFP.

To be able to understand the nature of zinc binding, blotting procedure was done on protein extracts with IAA alkylated cysteine residues and with the presence of reducing agent TCEP. As seen in Figure 3.21, proteins modified with IAA and membrane incubated in presence of TCEP did not stain for zinc.

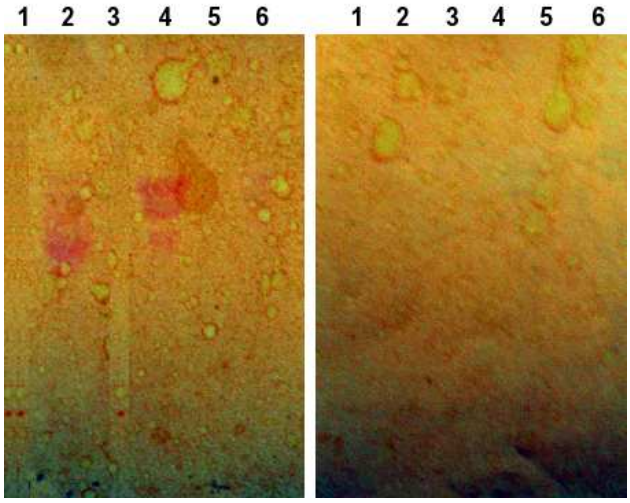

Figure 3.21: Effect of IAA and TCEP in the blotting procedure. Left and right membranes are identical. Left membrane was treated regularly, whereas right membrane was treated with the precence of 5 mM TCEP at all steps of zinc detection procedure.
Lane 1: Gliadin extraction from 6920 embryo.
Lane 2: Gliadin extraction from 6920 endosperm.
Lane 3: Glutenin extraction from 6920 embryo.
Lane 4: Glutenin extraction from 6920 endosperm.
Lane 5: Glutenin extraction from 6920 embryo, treated with IAA.
Lane 6: Glutenin extraction from 6920 endosperm, treated with IAA.

Increasing amounts of protein were loaded to gels and later blotted and stained for zinc to see the effect of protein amount on the blotting procedure and to see if there are zinc binding proteins present in low concentrations. Results may be seen on lanes 7 to 14 of Figure 3.22. Increasing the protein

amount increased the intensity of staining.

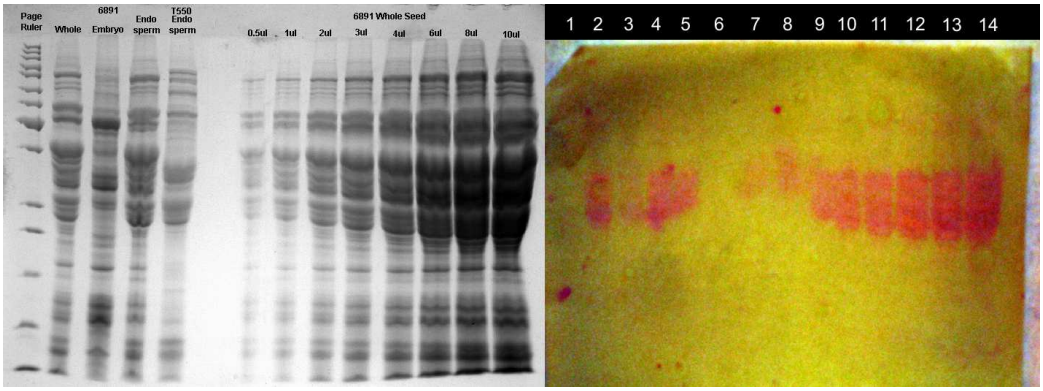

Figure 3.22: Effect of protein amount on detection of Zn bound to blotted proteins. Original gel is shown to the left.
Lane 1: PageRuler protein ladder.
Lane 2: Whole seed extract. Lane 3: Embryo extract.
Lane 4: Endosperm extract. Lane 5: type550 white flour extract.
Lanes 7–14: Whole seed extracts loaded 0.5 μl, 1 μl, 2 μl, 3 μl, 4 μl, 6 μl, 8 μl and 10 μl.

3.5 Microscopy

Longitudinal sections of seeds were stained with PAN and ninhydrin and results are shown in Figure 3.23. The backgrounds are yellow due to the color of PAN and ninhydrin solutions. Red regions in PAN staining are heavy metal containing parts, and purple regions in ninhydrin staining are high concentration protein containing parts.

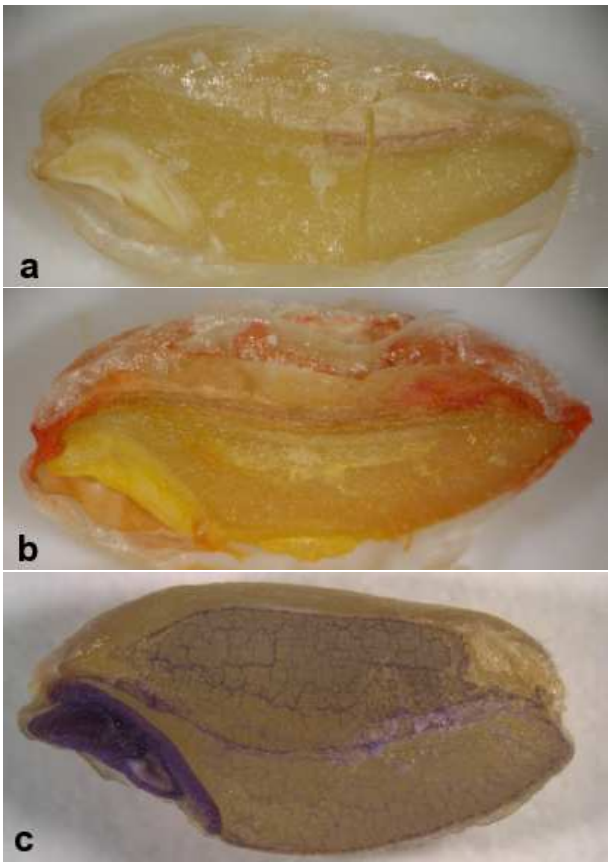

Figure 3.23: Seed longitudinal sections, non-stained (a) and stained with PAN (b) and ninhydrin (c).

Chapter 4

Discussion

4.1 Zinc and nitrogen applications have effects on wheat plant

Results of applications of different concentrations of zinc and nitrogen have been introduced in Section 3.1. It is seen from the data that zinc and nitrogen applications had effects on the dry weight and produced seed amounts, as well as effects on the accumulation of zinc, phosphor and nitrogen in the seed. Foliar applications of zinc and nitrogen in this trial did not present any significant effects.

At low applications of nitrogen (75 ppm), increasing the concentration of the zinc applications decreased the dry weight production, possibly due to zinc toxicity [137]. However, at 225 ppm nitrogen application, dry matter production was proportional to the concentration of zinc application (Figure 3.1). Similar results were also seen in the nitrogen accumulation in the seed. Except for 75 ppm nitrogen application, increasing zinc application, increased the accumulated nitrogen in the seed (Figure 3.2). This suggests that, zinc application has a positive effect on the growth of the plant and seed formation, which is consistent with the literature [138]. As zinc application shows toxicity at low nitrogen concentrations, but not when sufficient

nitrogen is present, mechanisms involving nitrogen, such as protein synthesis, might play a role in detoxification of zinc.

Increasing the nitrogen application increases the dry matter production to some extent (Figure 3.1). At nitrogen applications 675 ppm and higher, dry matter production shows a decrease. Significant differences in seed production at different growth conditions were not observed.

As noted in the literature, zinc accumulation in the seed was proportional to the zinc application (Figure 3.2) [138]. It is also observed that zinc accumulation is also proportional to the nitrogen application. The correlation between zinc and nitrogen accumulation to zinc and nitrogen application supports the idea that some proteins are sinks for zinc.

Phytates are also thought to be responsible of zinc accumulation [90]. For this reason, phophorus measurements were taken to see if there was a correlation between zinc application and phophorus accumulation (Figure 3.2). No correlation was observed. Nitrogen application of 675 ppm and above decreased the phosphorus accumulation in the seed, because high nitrogen present in the soil decreases the uptake of phosphorus [139].

4.2 DTZ and PAN solutions detect heavy metals

Heavy metal indicators, such as DTZ and PAN, have been used by environmental scientists to detect heavy metal contamination and its level in water and soil [140]. As discussed in Section 1.3.2, these chemicals have also been used to localize heavy metals in tissues. For these reasons, the properties of DTZ and PAN solutions were studied to see if these chemicals can be used in this research.

As seen in the photograph in Figure 3.3 and in spectrophotometric mea-

surements in Figures 3.4, 3.6 and 3.5, upon interaction with various heavy metals, these indicators change color dramatically, even in low concentrations of metal, and color change is proportional to the concentration. This suggests that these indicators not only may be used for detection of metals, but also for their quantification. In cases where only one metal is present, these indicators can also be used for identification, as with some metals, the color change is specific to that metal only.

In this research, these indicators were used to detect zinc in proteins. Although sensitivity of DTZ is higher compared to PAN, since DTZ also interacted with buffers used in experiments and since the concentrations of zinc encountered in this research were not that low, PAN was chosen for most of the applications. DTZ and PAN is not soluble in water and organic solvents are required to get a working solution. This incompability has created limitations of application.

4.3 Protein and zinc is more concentrated in seed embryo

It is already known that protein and zinc concentration is higher in embryo and aleurone layer than the starchy endosperm [115, 35]. This has also been observed during the experiments with seed section staining and protein extraction from tissues. As seen in the microscopy results in Figure 3.23, abundance of protein and zinc is much higher in the embyro when compared to the endosperm.

In Table 3.3, protein yields from embryo and endosperm tissues are shown. In all cases, extractions were first attempted with the same proportion of solvent to meal to facilitate comparison of concentrations of extracts directly. However, as results from seed section experiments were reviewed and it was

seen that protein concentrations were much higher in the embyro, the solvent to meal ratio was increased to avoid saturation. For the comparison of the extraction yields, differences in dilutions were taken into account and values were normalized. As expected, embryo total extract has more protein than endosperm total extract. The differences between the protein profiles will be discussed below.

4.4 Proteins of different extraction methods

In this research, mainly two methods were used to extract proteins. These were Osborne fractionation and extraction for SDS-PAGE analysis. The extract for SDS-PAGE analysis includes all the proteins from different Osborne fractions. This method yields a profile of the total protein extraction. Since the method of Osborne separated different seed proteins into fractions, it was possible to identify some of the seed proteins.

In the original method, Osborne fractionation gives four protein containing supernatants, as mentioned in Section 2.2.4, however, after preliminary results, fractionation was halted after propanol solution extractions. It was observed (Figure 3.8) that protein profile of SUP5 resembles the protein profiles acquired by total extraction. Recommended procedures on the supernatants were also not followed, as explained below.

When recommended procedures [127] were applied, most of the proteins were lost due to precipitation or were diluted during dialysis steps. To maintain the protein yield, proteins were kept in the original extraction buffer, which in turn prevented lyophilization. Thus, extracts could not be concentrated and samples could be preserved without degradation or aggregation only for short periods. Most reliable results were obtained in the analyses shortly after extractions.

Also, Osborne fractionation protocol states that each extraction step should be repeated two times and that the supernatants should be combined. However, since samples could not be lyophilized, combining supernatants only diluted the extracts. In Table 3.3, differences in the protein concentration of repeats of extraction can be seen. Extraction steps were repeated to remove all the proteins, but the second supernatants were discarted.

Native extraction was tried, but this method was not pursued, when it was seen that the profile of the native extract is actually very similar to SUP2–3 of Osborne fractionation.

A modification to Osborne procedure, suggested by Singh *et al.* [128], was also used to extract gliadins and glutenins. Results of this extraction (Figure 3.13) were compared to the Osborne fractions (Figure 3.12) and it was observed that the extracts of Singh corresponded to the supernatants SUP4a and SUP4b. SUP4a contains monomeric prolamins. Polymeric prolamins, held together with disulphide bonds, are not readily soluble in 50% propanol, therefore reducing agents should be included in the extraction solution to break the disulphide bridges and have these proteins in solution. SUP4b contains these polymeric prolamins. According to Fido *et al.* [127], glutenins, glutelins of wheat, are extracted with the borate buffer and are present in SUP5. Protein profile of SUP5 is observed to be different from glutenin extracts of Singh method. This inconsistency should be noted here.

Although the Osborne procedure facilitated separation of seed proteins into different fractions, each fraction still contained several different proteins (subunits). This made use of native gel electrophoresis ineffective as can be seen in Figure 3.14.

Seed protein extracts were separated on SDS-PA gels by electrophoresis. For the purpose of this analysis, a concentration of 12% PA was adequate,

when completely polymerized giving good resolutions both for high molecular weight and low molecular weight proteins.

Because there are a large number of proteins in seeds, and because protein(s) of interest was (were) not yet identified, chromatography was not used.

4.5 Different tissues of the seed have different protein profiles

Extractions from embryo and endosperm tissues showed that, these tissues have different protein profiles. From the data in Table 3.3, it is seen that embryo has an abundance of water and mild saline soluble proteins, whereas alcohol soluble storage proteins are abundant in the endosperm. This was expected, since embryo has most of the metabolic activity and cytoplasmic proteins are water or mild saline soluble proteins, whereas starchy endosperm contains polymeric storage proteins, insoluble in water and mild saline.

Major differences between high and low zinc and nitrogen content seed were not observed, but there are some proteins that need further attention. As the proteins were loaded at normalized amounts, and staining level of some major bands were similar, protein bands of different staining levels are likely to be due to a difference in levels of expression in the presence of zinc or nitrogen. In Figure 3.12, a band of 30 kDa of NaCl and total extractions of endosperm shows increased levels in high content seeds. There is another significant band in the NaCl extraction of embryo between 50 kDa and 60 kDa that shows to be more in high content seeds. However, these results are highly speculative.

4.6 Developed blotting method detects zinc binding on PVDF membranes

A new method was developed from Mazen *et al.* [119] and was used to detect if certain proteins were able to bind zinc. There were numerous studies in literature that made use of the original method [103, 141, 142, 143], however, since this method utilized radioactive isotope of zinc, it was modified to use non-radioactive zinc. Also, this method has not been used on seed proteins, previously. The validity of this new method was checked extensively.

After the first results that showed staining (Figure 3.16), the nature of this staining was investigated. Protein blotted PVDF membranes were incubated in solutions containing no metals, but EDTA to chelate any contaminating metals and no staining was seen (Figure 3.19), suggesting that the staining is due to zinc, not due to the chemistry of proteins at the stained bands. Similar stainings were acquired from experiments where the protein blotted membranes were incubated in solutions containing different concentrations of $ZnCl_2$ (Figure 3.18), suggesting that zinc concentration is not a determining factor for the staining. For the specificity of the binding, lower concentrations of zinc was chosen in incubations.

Effect of cadmium was also checked. When compared to the staining after zinc incubation, cadmium incubated membranes showed a lesser degree of staining (Figure 3.20). This suggested that the ligands are more specific to zinc.

Membranes were blotted with GST-dMT and BSA that are known to bind zinc [135, 136], and the procedure was repeated, showing staining at GST-dMT and BSA bands (Figure 3.19). Increasing amounts of loaded proteins showed more intense staining (Figure 3.22). This correlation between the amount of zinc and the amount of protein shows that stained zinc is bound

to proteins, and when more protein is present, more zinc is able to bind and thus more staining is achieved.

When zinc incubation was done in presence of the reducing agent TCEP, no staining was seen. Similarly, proteins with modified cystine residues with IAA showed no staining (Figure 3.21). TCEP when present in the incubation, constantly reduces the cysteine residues and IAA S-carboxymethylates the cysteine residues, irreversibly [129]. Distruption of staining after modification of cysteine residues and under constant reducing suggests that zinc binding is faciliated by the cystine residues.

Proteins that are separated by SDS-PAGE are blotted on PVDF membranes, and are later monitored for zinc. These proteins are denatured and are allowed to renature in the renaturation buffer. It is not clear to what extent the proteins are able to refold to their native structure, if at all, but some renaturation seems to occur that faciliates zinc binding. The procedure works better when Triton X-100 is included in the renaturation buffer. Triton removes SDS more effectively than the plain buffer, therefore providing a more suitable environment for proteins to renature and giving better results. Use of Triton X-100 to renature proteins is present in the literature [144].

Various methods to stain the membranes were tried. Staining the membranes in metal indicator solutions were not possible, because bound zinc was chelated by the dye in the solution immidiately before any photos could be taken. For this reason, staining was done by spraying metal indicator solutions on the membranes, not distrupting the localization of zinc. Initially, DTZ was used in staining procedures, although it also interacted with the buffer. Later, DTZ was replaced with PAN, because PAN was more specific to zinc, and less sensitive than DTZ, so that the background was reduced and did not interact with buffers used in the procedure.

4.7 Zinc binding was detected on some proteins on PVDF membranes

During the validation of the method, samples were chosen among protein extracts of greenhouse samples. Total extractions, Osborne fractions and Singh fractions from whole seed, embryo and endosperm sections were analyzed for zinc binding proteins.

Experiments gave the following results:

1. Some proteins blotted on the membranes are stained for zinc after the procedure.

2. These proteins belong to the Osborne fractions SUP4a and SUP4b. Osborne fractions SUP4a and SUP4b are prolamins abundant in the starchy endosperm. From SDS-PAGE analysis, it is seen that these stained proteins have molecular weights between 30 kDa and 50 kDa.

3. Comparing above results with the literature on seed storage proteins presented in Section 1.2.4, these proteins are thought to be sulfur rich low molecular weight prolamins. Sulfur is located in cysteine residues of the proteins.

4. These result are consistent with the results of experiments with IAA and TCEP. Zinc binding is said to be faciliated by cysteine residues, as discussed earlier.

5. Procedure on total extracts gave similar results.

6. With this detection method, no proteins from embryo extracts were stained on PVDF membranes and this contradicts literature [115].

7. This surpising result may be interpreted as (a) embryo stores Zn in non-peptide complexes e.g. in phytate or in a form which is completely destroyed by our procedures, or (b) Zn-binding proteins of the embryo are hard to detect by electrophoresis due to their low molecular weight. These proteins may also exist in such low quantities and may also be so instable that the method is not sensitive enough to detect them.

8. No differences were detected between high and low zinc and nitrogen containing seeds.

Chapter 5

Conclusion

Both zinc and nitrogen applications have positive effects on the dry weight and on the accumulation of nitrogen and zinc in the seed.

There are differences between embryo and endosperm tissues of the seed in terms of protein concentration, protein profile and zinc concentration. Protein and zinc concentration is higher in the seed embryo. Polymeric storage proteins are majorly found in the starchy endosperm, whereas albumins and globulins are located in the embryo.

With the developed zinc detection method, it is showed that sulfur rich LMW (M_r: 30–50 kDa) prolamins bind zinc via cysteine residues on PVDF membranes, however it is not known what happens *in vivo*.

Contrary to the literature, no zinc binding proteins were detected on extracts from embryo tissues. This may be due to the fragility, the size and the concentration of those proteins, if they exist.

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Appendix A

Additional Data

Table A.1: Total dry matter (TDM) and seed production of individual samples. Values are one plant averages from each pot. Total dry matter weight is the total weight minus seed weight, after complete drying in the oven. Growth conditions are indicated in the labels.

	75 ppm N			**675 ppm N**		
	Sample	Seed (g.)	TDM (g.)	Sample	Seed (g.)	TDM (g.)
0.5 ppm Zn	6864	1.31	2.24	6894	1.71	2.14
	6865	1.37	1.96	6895	2.88	3.18
	6866	1.16	1.76	6896	1.57	2.28
	6867	1.37	2.39	6897	2.29	2.53
	6868	1.52	2.33	6898	1.92	2.17
8.0 ppm Zn	6869	1.24	1.63	6899	1.84	2.47
	6870	1.83	2.17	6900	1.81	2.43
	6871	1.33	1.72	6901	2.08	2.28
	6872	1.28	1.69	6902	2.33	2.42
	6873	1.12	1.38	6903	1.85	2.21
24.0 ppm Zn	6874	1.30	1.66	6904	1.67	2.32
	6875	1.59	2.01	6905	2.06	2.61
	6876	1.52	1.94	6906	1.49	2.07
	6877	1.24	1.48	6907	1.37	1.93
	6878	1.31	1.52	6908	1.59	2.19

	225 ppm N			**675 ppm N + foliar**		
	Sample	Seed (g.)	TDM (g.)	Sample	Seed (g.)	TDM (g.)
0.5 ppm Zn	6879	1.75	2.10	6909	1.70	1.87
	6880	1.51	2.17	6910	2.13	2.64
	6881	1.45	2.89	6911	1.97	2.69
	6882	1.58	2.32	6912	1.81	2.42
	6883	1.47	2.60	6913	1.93	1.95
8.0 ppm Zn	6884	1.88	3.42	6914	1.72	2.49
	6885	1.62	2.78	6915	1.77	2.53
	6886	1.73	2.51	6916	2.21	2.25
	6887	1.81		6917	1.86	2.20
	6888	1.68	3.06	6918	1.79	2.91
24.0 ppm Zn	6889	1.99	3.84	6919	1.72	2.01
	6890	1.66	2.80	6920	1.71	2.25
	6891	1.18	2.54	6921	2.04	2.59
	6892	1.64	3.36	6922	1.33	2.22
	6893	1.33	2.74	6923	1.32	2.07

Table A.2: Zinc and phosphorus accumulation in the seeds of individual samples. Growth conditions are indicated in the labels.

	75 ppm N			675 ppm N		
	Sample	Zn (ppm)	P (ppm)	Sample	Zn (ppm)	P (ppm)
0.5 ppm Zn	6864	17	4977	6894	19	4646
	6865	20	5191	6895	12	4041
	6866	18	4904	6896	17	4843
	6867	17	5014	6897	14	4150
	6868	18	4941	6898	17	4379
8.0 ppm Zn	6869	61	5136	6899	84	4577
	6870	66	5076	6900	68	4358
	6871	61	4835	6901	71	4119
	6872	62	4992	6902	71	4004
	6873	60	5141	6903	76	4185
24.0 ppm Zn	6874	72	4903	6904	97	4341
	6875	70	4784	6905	84	3721
	6876	68	4782	6906	91	4368
	6877	92	5339	6907	102	4856
	6878	82	5053	6908	98	4454

	225 ppm N			675 ppm N + foliar		
	Sample	Zn (ppm)	P (ppm)	Sample	Zn (ppm)	P (ppm)
0.5 ppm Zn	6879	17	5207	6909	17	4326
	6880	15	5195	6910	18	4440
	6881	13	4763	6911	19	4262
	6882	15	4867	6912	15	3964
	6883	21	5311	6913	15	4121
8.0 ppm Zn	6884	73	4645	6914	76	3919
	6885	82	5005	6915	79	4627
	6886	74	5039	6916	77	4612
	6887	82	5047	6917	70	4204
	6888	86	5173	6918	79	4252
24.0 ppm Zn	6889	99	5248	6919	89	4399
	6890	104	5206	6920	125	5206
	6891	127	5496	6921	86	4216
	6892	110	5225	6922	99	4478
	6893	111	5173	6923	105	4644

Table A.3: Nitrogen accumulation and estimated grain protein content (GPC) in the seeds of individual samples. GPC is calculated from nitrogen data, by multiplying with a factor of 5.83. Growth conditions are indicated in the labels.

	75 ppm N			**675 ppm N**		
	Sample	N (%)	GPC (%)	Sample	N (%)	GPC (%)
0.5 ppm Zn	6864	2.57	15.00	6894	3.07	17.90
	6865	2.68	15.62	6895	3.26	19.00
	6866	2.60	15.18	6896	3.35	19.54
	6867	2.72	15.85	6897	2.90	16.89
	6868	2.79	16.29	6898	2.99	17.45
8.0 ppm Zn	6869	2.76	16.09	6899	3.16	18.39
	6870	2.25	13.09	6900	3.03	17.65
	6871	2.57	14.97	6901	3.20	18.68
	6872	2.45	14.30	6902	3.22	18.78
	6873	2.60	15.13	6903	3.13	18.27
24.0 ppm Zn	6874	2.69	15.66	6904	3.19	18.62
	6875	2.31	13.44	6905	3.30	19.21
	6876	2.30	13.39	6906	3.33	19.41
	6877	2.72	15.87	6907	3.18	18.54
	6878	2.44	14.22	6908	3.19	18.58
	225 ppm N			**675 ppm N + foliar**		
	Sample	N (%)	GPC (%)	Sample	N (%)	GPC (%)
0.5 ppm Zn	6879	3.12	18.17	6909	3.11	18.12
	6880	3.05	17.76	6910	3.12	18.20
	6881	2.98	17.39	6911	3.15	18.34
	6882	2.96	17.27	6912	3.10	18.06
	6883	3.09	18.04	6913	3.09	17.99
8.0 ppm Zn	6884	3.32	19.38	6914	3.20	18.67
	6885	3.22	18.75	6915	3.15	18.39
	6886	3.22	18.76	6916	3.09	18.01
	6887	3.28	19.11	6917	3.22	18.76
	6888	3.28	19.11	6918	3.19	18.59
24.0 ppm Zn	6889	3.43	19.98	6919	3.05	17.77
	6890	3.37	19.67	6920	3.45	20.09
	6891	3.50	20.42	6921	3.38	19.70
	6892	3.16	18.41	6922	3.52	20.54
	6893	3.19	18.61	6923	3.15	18.37

Appendix B

Materials

B.1 Solution recipes

SDS extraction buffer 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, 0.002% (w/v) bromophenol blue in ddH_2O.

2x Laemmli sample buffer 125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue in ddH_2O.

2x Native sample buffer 200 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue in ddH_2O.

SDS-PAGE running buffer 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS in ddH_2O.

Native-PAGE running buffer 25 mM Tris, 192 mM glycine in ddH_2O.

Coomassie staining solution 0.1% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid in ddH_2O.

Reducing buffer for blotting SDS-PAGE running buffer, 0.7 M 2-mercaptoethanol in ddH_2O.

SDS transfer buffer SDS-PAGE running buffer, 20% (v/v) methanol in ddH_2O.

Native transfer buffer 100 mM CAPS buffer pH 11, 10% methanol in ddH_2O.

Renaturation buffer 50 mM Tris-HCl pH 7.5 in ddH_2O.

Zinc incubation buffer 50 mM Tris-HCl pH 7.5, 100 mM KCl in ddH_2O.

Metal indicator solutions 2 mM PAN or DTZ in 100% acetone.

B.2 Chemicals

Chemical	Company	Catalog No.
Acetic acid (glacial)	Riedel-de Haën, Germany	27225
Acetone	Merck, Germany	100013
30% Acrylamide-0.8% Bi-acrylamide	Sigma, Germany	A3699
Albumin (bovine serum)	Sigma, Germany	A7906
Ammonium persulphate	Carlo-Erba, Italy	420627
Boric acid (99%)	Sigma, Germany	B6768
Bradford reagent	Sigma, Germany	B6916
Bromophenol blue	Applichem, Germany	A3640
1-Butanol	Merck, Germany	100988
Cadmium (II) sulphate	Fluka, Switzerland	20920
Calcium nitrate	Merck, Germany	102120
Copper (II) sulphate	Riedel-de Haën, Germany	12849
Coomassie Brilliant Blue R-250	Fluka, Switzerland	27816
N-Cyclohexyl-3-aminopropanesulfonic acid	Amresco, USA	0365
ddH_2O	Millipore, France	
Didifos 55 EC	Hektas, Turkey	
Dithiocarb	Sigma, Germany	D3506
1,4-Dithiothreitol	Fluka, Switzerland	43815
Dithizone	Merck, Germany	103092
DryEase mini cellophane	Invitrogen, Germany	NC2380
Ethanol	Riedel-de Haën, Germany	32221
Ethylenediaminetetraacetic acid	Riedel-de Haën, Germany	27248
Ferric EDTA	Fluka, Switzerland	03650
Glycerol (87%)	Riedel-de Haën, Germany	15523
Glycine	Amresco, USA	0167
Hybond-P PVDF membranes	Amersham Biosciences, Sweden	RPN2020F
Hydrochloric acid (37%)	Merck, Germany	100314

Chemical	Company	Catalog No.
Hydrogen peroxide (30%)	Merck, Germany	107209
Iodoacetic acid	Merck, Germany	822282
Lead (II) nitrate	Riedel-de Haën, Germany	11520
Magnesium sulphate	Riedel-de Haën, Germany	13246
2-Mercaptoethanol	Aldrich, Germany	M370-1
Mercury (I) chloride	Fluka, Switzerland	83354
Methanol	Riedel-de Haën, Germany	24229
Nickel (II) chloride	Riedel-de Haën, Germany	31462
Ninhydrin	Fluka, Switzerland	72490
Nitric acid (65%)	Merck, Germany	100456
PageRuler protein ladder	Fermentas, Germany	SM0661
Phenylmethylsulphonyl-fluoride	Amresco, USA	0754
Ponceau S	Applichem, Germany	A1405
Potassium chloride	Fluka, Switzerland	60129
Potassium dihydrogen phosphate	Riedel-de Haën, Germany	04243
Potassium sulphate	Merck, Germany	105153
Prestained protein MW marker	Fermentas, Germany	SM0441
1-Propanol	Merck, Germany	100996
Protein assay	BioRad, USA	500-0006
1-(2-Pyridylazo)-2-naphthol	Fluka, Switzerland	82960
Sodium chloride	Riedel-de Haën, Germany	13423
Sodium dodecyl sulphate	Sigma, Germany	L-4390
Tetramethylethylenediamine	Sigma, Germany	T-7029
Tris	Fluka, Switzerland	93349
Tris(2-Carboxyethyl) Phosphine, HCl	Sigma, Germany	C4706
Triton X-100	Applichem, Germany	A1388
Urea	Fluka, Switzerland	51461
Zinc (II) chloride	Riedel-de Haën, Germany	14422
Zinc (II) sulphate	Merck, Germany	108883

B.3 Equipment

Autoclave Hirayama, Hiclave HV-110, Japan.

Balance Sartorius, BP610, BP221S, BP221D, Germany.

Blot Module Novex, X Cell II Blot Module, USA.

Centrifuge Eppendorf, 5415R, Germany; Hitachi, Sorvall RC5C Plus, USA.

Cuvette Hellma, QH, QS, Germany.

Deep Freeze Bosch, -20°C, Turkey.

ddH_2O Millipore, MilliQ Academie, Elix-S, France.

Digital Camera Canon, PowerShot SD 400, USA; Olympus, C-7070, USA.

Electrophoresis BioRad Inc., USA; Novex, X Cell SureLock Electrophoresis Cell, USA.

Element Analysis Varian, Vista-Pro CCD Simultaneous ICP-OES, Australia; LECO, TruSpec CN, USA.

Gel Documentation BioRad, Universal Hood II, USA; BioRad, Quantity One, USA; BioRad, GelDoc XR, USA.

Gel Dryer EC Apparatus Corporation, Gel Dryer EC355, Gel Dryer Pump EC353, USA.

Ice Machine Scotsman Inc., AF20, USA.

Imaging Software GIMP 2.2.12.

Lighting Olympus, LG-PS2, USA.

Magnetic Stir VELP Scientifica, ARE Heating Magnetic Stirrer, Italy.

Microliter Pipette Gilson, Pipetman, France.

Microplate Reader BioRad, Model 680 Microplate Reader, USA.

Microscope Olympus, SZ61, USA.

Microwave Oven CEM Corp., Mars Xpress, USA; Bosch, Turkey.

pH Meter WTW, pH540GLP MultiCal, Germany.

Power Supply BioRad, PowerPac 300, USA; Wealtec, Elite 300, USA.

Refrigirator Bosch, +4°C, Turkey.

Sonicator Bioblock Scientific, Vibracell 75043, France; Bandelin, Sonorex, Germany.

Spectrophotometer Schimadzu, UV-3150, Japan; Nanodrop, ND-1000, USA.

Thermomixer Eppendorf, Thermomixer Comfort, Germany.

Vortex VELP Scientifica, $2x^3$, Italy.