CHAPTER 19

Micronutrients

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Little effort has been made to apply modern breeding techniques to adapt crop plants to soils of poor nutritional status, even though this is genetically feasible. Rather, the use of fertilizers to solve soil nutrient problems agronomically has encouraged plant breeders to concentrate on other objectives such as yield, climatic adaptation, disease resistance, and quality.

Some nutritional problems, however, do not appear to be easily resolved, and a case for breeding adapted varieties can be made in those instances. The most obvious problems are those of nutrient toxicities, where the cost of removal is much greater than that of applying fertilizer to address deficiencies; hence breeding solutions are more practical and indeed necessary. Furthermore, fertilizers are ineffective with micronutrient deficiencies such as iron and manganese, which are induced by high pH; agronomic solutions may thus not be satisfactory, and a genetic solution is necessary.

Soils with chronic micronutrient deficiencies are often high-pH, calcareous soils in seasonally dry climates, but may include deep sands in any climate. Although micronutrient fertilizers are often strikingly beneficial, the plant's yield potential can only be reached if its roots penetrate the chemically inhospitable subsoils to access water stored there. Tolerance to micronutrient deficiencies (that is, micronutrient efficiency; see definition below) is generally manifested by greater uptake from deficient soil. Roots must find micronutrients in their immediate environment to continue to grow, to express disease resistance, and to access water stored in the subsoil.

In this chapter we outline the case for breeding crop plants for traits that confer adaptation to nutrient deficiencies in soils. In this context a deficient soil is one that contains reasonable amounts of the limiting nutrient; however, it is relatively unavailable to the common cultivars of the crop in question. We then discuss the genetics of micronutrient traits and the status of screening techniques that can be used in breeding programs.

Definition of Nutrient Use Efficiency

We define nutrient use efficiency (for each element separately) as a genotype's ability to produce high yield in a soil whose nutrient content is limiting for a standard genotype. This agronomic definition is meaningful to a plant breeder selecting genetic material in the field. Often a nutrient-efficient genotype in infertile environments will also have high yield potential in fertile soils. Nutrient efficiency may be achieved physiologically by one or more mechanisms:

- better root system geometry;
- faster specific rate of absorption at low concentrations (low K_m);
- chemical modification of the root-soil interface to solubilize more of the limiting nutrient;
- improved internal redistribution of the nutrient;
- superior nutrient utilization, or a lower functional requirement for the nutrient in the cell.

In the field the plant breeder cannot readily identify the operative mechanism(s), but selection would be more precise if he/she knew which ones they are. Examples of this are given in the text, but for the greater part, efficiency is inferred from yield measurements, nutrient content, or symptom expression.

The Case for a Breeding Program

Plant breeding is a numbers game, and any new objective, such as micronutrient use efficiency, represents a considerable escalation of the breeder's work or else a diversion of effort away from traditional targets such as quality and disease resistance. Thus there must be a strong case for a new breeding objective before it can be added to the research agenda. The lack of compelling arguments is the reason little effort has been made until

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now to adapt crop plants to micronutrient-deficient soils. To argue for breeding to improve nutritional characters, it is necessary to show:

- a need for it as pressing as for other breeding objectives;
- there is reasonable genetic potential to be exploited;
- it is agronomically, economically, and ecologically feasible.

Graham (1984, 1987, 1988a, b) demonstrated that there is genetic diversity for micronutrient characters within wheat, and further argued that nearly all soils, no matter how poor, had sufficient amounts of micronutrients stored in the profile. The problem was usually their lack of availability due partly to soil chemistry, but equally to poor genotypic adaptation. This brings us back to the agronomic arguments.

Twelve years ago, Ascher and Graham (1993) bulldozed off the topsoil and dug up the subsoil from grave-sized pits on 10 soil types scattered across South Australia. Various nutrient treatments were applied to the subsoils as they were returned to the pits in their original layers. The topsoils were then replaced and the sites sown by each farmer as part of the field. The farmers applied all the usual treatments and fertilizers, including in some cases micronutrients, without disturbing the treated subsoil below. Responses to the nutrients were immediate and often spectacular, though in general there was little response to physical disturbance only or to gypsum, which underlines the chemical nature of the problem.

It is important to note that these responses (at the five most trace-element-deficient sites) continue to the present, and with the original nitrogen most likely lost by now, the residual responses are probably due to

phosphorus and trace elements (Picture 1). Indeed, the micronutrient treatment seems to have relatively greater residual value as time passes. It appears that root channels developed to depth in the early years have been re-utilized annually to the present. In pot studies we have shown that wheat roots grow poorly in subsoil even when fertilized with nitrogen and phosphorus. Although we are experimenting with methods of deep placement of micronutrients with highly unconventional and expensive machinery, we believe a better approach to this problem is to breed wheats with roots that will penetrate subsoils with low availability of phosphorus and micronutrients.

Immediately relevant to this argument is the picture emerging from physiological studies of roots spanning four decades. From the papers of Haynes and Robbins (1948), Epstein (1972), Pollard et al. (1977), Bowling et al. (1978), Graham et al. (1981), Welch et al. (1982), Nable and Loneragan (1984), Loneragan et al. (1987) and Holloway (1991), it appears that the elements phosphorus, zinc,

boron, calcium, and manganese are all required in the immediate external root environment for healthy growth, membrane function, and cell integrity. In particular, phosphorus and zinc deficiencies in the external environment promote leaking of cell contents such as sugars, amides, and amino acids (Graham et al., 1981), which are chemotactic stimuli to pathogenic organisms.

Phosphorus is phloem mobile, but the other elements are not, or are poorly so; this means that the root tips cannot be adequately supplied from elsewhere in the root system, such as for example, from roots that come into contact with a fertilizer band. Moreover, in the case of zinc, a high internal zinc content did not prevent leakiness due to a deficiency of zinc external to the membrane (Welch et al., 1982). It follows that the roots of those wheat genotypes that have a greater capacity to mobilize nutrients strongly bound to soil particles in the rhizosphere will be better able to penetrate the infertile, high pH subsoil. It follows too from the above that roots

Picture 1. Grave-sized barley plots in a farmer's field at Marion Bay, 1992. The areas around the graves represent completely undisturbed subsoil. The middle plot shows a huge response to subsoil N, P, and micronutrients, even after seven seasons and despite regular topsoil fertilization by the farmer. (Photo: J. Ascher-Ellis.)

that are far from a fertilizer band and have leaky membranes are at greater risk from pathogens.

Recent studies have clearly linked trace element deficiencies with enhanced susceptibility to particular pathogens (Graham and Webb, 1991). Manganese-deficient wheat plants are more susceptible to *Gaeumannomyces graminis* var. *tritici*, the take-all fungus (Graham and Rovira, 1984; Huber and Wilhelm, 1988), and to the foliar pathogen, *Erysiphe graminis* (Graham, 1990). Zinc deficiency decreased the resistance of wheat to *Fusarium graminearum,* the crown rot fungus (Sparrow and Graham, 1988) and to *Rhizoctonia solani* (Thongbai et al., 1993a, b), the causal agent of bare patch. The implication is that nutrient-efficient genotypes growing in deficient soil, enjoy better nutrient status and should have greater resistance to these pathogens; this has been confirmed by Wilhelm et al. (1990), Pedler (1994) and Rengel et al. (1993) for the manganese efficiency /take-all system and by Grewal et al. (1996) for the zinc efficiency/crown rot system. Collectively, these results suggest causality in the concurrence in South Australia of some of the world's most severe root disease and micronutrient deficiency problems.

Topsoil drying also affects wheat production on infertile soils of the seasonally humid zone by causing loss of fertilizer efficiency. Most micronutrients are in the topsoil by virtue of fertilizer additions and nutrient cycling. Leaching of the heavy metals is negligible (Jones and Belling, 1967). When the topsoil dries as a result of a week or two of dry weather in spring, roots in the nutrient zone are largely deactivated and the plant must rely on deeper roots or retranslocation for further nutrition. With phloem-immobile

micronutrients and inefficient genotypes, deficiency can result. This occurs in the field (Grundon, 1980), where copper deficiency from topsoil drying at early boot stage can cause severe sterility problems; similarly in a pot trial Ascher and Graham (see Table 5, Graham, 1990). These authors showed that Cu deficiency could be induced in wheat plants growing in tall pots if an otherwise adequate Cu supply in the topsoil was rendered unavailable by soil drying and there was insufficient available Cu in the subsoil. This problem may, however, be overcome by using copper-efficient genotypes such as triticale (Grundon and Best, 1981).

Two further advantages accrue to micronutrient efficient varieties if by virtue of their efficiency they also accumulate more of the limiting nutrient in the grain: 1) they improve human nutrition if consumed (iron, zinc, especially), and 2) seedling vigor is markedly better when the seed is resown on deficient soils. A final advantage: the degree of micronutrient efficiency currently known to exist in wheat germplasm, if deployed in modern varieties, would overcome subclinical deficiency commonly unrecognized by farmers or their advisers.

The Case for High Nutrient Reserves in Seed

Reserves of nutrients in the seed must be sufficient to sustain growth until the root system has developed sufficiently to supply nutrients from the growth medium. During plant establishment nutrients are supplied partly from seed reserves and partly from the soil. High levels of seed nutrients are particularly important in soils with low nutrient

availability, since a larger root system is required for the soil to supply the needs of the crop. Low nutritional status of seeds has been reported to reduce plant growth under conditions of low nutrient availability (Marcar and Graham, 1986; Asher, 1987; Rengel and Graham, 1995a, b; Moussavi-Nik, 1997).

The nutrient status of seed has been shown to affect both seed viability and seedling vigor. There are reports in the literature of minimum seed concentrations of nutrients below which seedlings will not grow normally (Ascher and Graham, 1993). Seedling nutrition has been shown to be an important factor in plant susceptibility to pathogens (Graham, 1983; Graham and Webb, 1991; Pedler, 1994; Streeter, 1998); seed nutrient levels that fail to maintain adequate nutritional status of seedlings in infertile soils may reduce the plant's resistance to some seedling diseases. The importance of seed nutrient status in confounding the screening procedures for micronutrient use efficiency traits is dealt with in a later section.

Mineral Nutrient Quality of Grain

The mineral content of grain contributes not only to seedling vigor in the next generation but—equally important—to the nutrition of humans and animals. This is particularly so for micronutrients, since over half of the world's population is deficient in micronutrients, and cereal grains, the major component of the diet of those at risk, also contribute most of the minerals in their diet. Of primary concern in human nutrition are iron and zinc, and secondary concerns are provitamin A and the minerals iodine, calcium, selenium and copper (Graham and Welch, 1996).

Zinc is an element of special interest as it is commonly deficient in soils (50% globally), plants, animals, and humans.

Much can be achieved through the whole food chain by fertilizing the soil with zinc (Graham and Welch, 1996), but because of topsoil drying and the role of subsoil fertility, the case has been made elsewhere in this chapter for breeding zinc-efficient wheats, probably involving three or four genes per genome.

Where zinc efficiency translates into higher zinc concentration in the seeds, as it can, we have a win-win benefit for producers and consumers, and an additional mechanism for better yields in subsequent crops on deficient soils: zinc-dense seed. In some cases, however, zinc-efficient cultivars may enhance yield so much that the concentration of zinc in grain is diluted by extra dry matter (Graham et al., 1992). The evidence available indicates there are genes (independent of those controlling the agronomic zinc efficiency trait) that control the transport of zinc to the grain from vegetative parts during grain filling. Since the heavy metal nutrient cations iron, zinc, copper, manganese, cobalt, and nickel are insoluble at the high pH of the phloem sap, these transport genes probably code for natural chelators that hold these metals in solution in the phloem sap so they may be transported to the grain. One such gene, identified in tomato, codes for the synthesis of nicotianamine, which is essential for the transport of iron in that plant. In a nicotianamine-deficient recessive mutant, Chloranova, this trait is controlled at a single locus (Ripperger and Schreiber, 1982).

Genetic variation for iron and zinc concentration in grain has been explored in wheat by Ortiz-Monasterio and associates at CIMMYT. The range in concentration from the lowest to the highest for both elements is 3-5, and there is a potential advantage over current high yielding varieties of a factor of 1.5-3 (Graham et al., 1997). Although

high levels of iron and zinc are not co-inherited, there are genotypes higher in both, and among them are high yielding advanced lines.

Household resource allocation studies (Bouis, 1994) suggest that doubling the iron content in grain would significantly improve iron deficiency in humans, provided the iron in iron-dense varieties was bioavailable. The bioavailability of the iron in high-iron beans (and rice) has already been tested on iron-deficient rats and proven to be as available (percent of total) as that in low or standard types (Welch et al., 1999). Tests are under way in humans. Since beans are high in absorption inhibitors such as phytate and tannins, it is likely that an equally favorable result will be established for wheat in the near future.

Genetics of Micronutrient Traits

The first genetic study of a micronutrient efficiency factor was conducted by Weiss (1943). He showed that iron efficiency in soybeans was due to a single dominant gene that controls the reducing power of the root surface. Since this pioneering study, several minor additive genes have been discovered to contribute to iron efficiency in soybeans (Fehr, 1982). This situation of one major and several minor genes is generally the case with the micronutrients. Reports to about 1970 were reviewed by Epstein (1972), who noted that boron efficiency was apparently under simple genetic control in tomato and celery, as were iron efficiency in maize and tomato, and magnesium efficiency in celery. More recently iron efficiency in tomato has been shown to be controlled by a major gene, coding for an iron-transporting amine, nicotianamine, and a string of minor genes (Brown and Wann, 1982; Ripperger and Schreiber, 1982).

Copper efficiency

Copper efficiency in rye appears to be a dominant trait controlled at a single locus on the long arm of chromosome 5R (Graham, 1984). Copper efficiency has been transferred from rye to wheat by translocating part of 5RL to a chromosome of wheat. The translocated 5RL chromosome segment carrying the copper-efficiency trait Ce-1 onto the 4Aβ (now 4Bβ) chromosome confers on plants a much greater ability to mobilize and absorb copper ions tightly bound to the soil (Graham, 1984). Several such translocations exist but the 5RL/4A translocation appears to be the most satisfactory agronomic type (Picture 2); it has been successfully incorporated into

Picture 2. The copper-efficient 5R/4A plants on the right grow well and show good seed set in soil that is too copper-deficient for the control genotype (2R/4A).

cultivars adapted to South Australia (Graham et al., 1987). The 5RL chromosome arm also confers copper efficiency in triticale, unless a copper-inefficient rye is used in the cross. Triticales generally show agronomically useful copper efficiency, intermediate between wheat and rye, and have been used for this reason on many sandy or peaty copper-deficient soils in South Australia (Graham, 1987), and on deficient clayey soils in Queensland to counter the effects of topsoil drying (Grundon, 1980).

Work with these 5R materials has shown that copper efficiency in rye is not clearly linked to zinc or manganese efficiency. Thus independent and relatively specific genes are involved, and neither root system geometry nor size appears to be critical (Holloway, 1996).

Although rye has a much longer and finer root system than wheat, triticales generally do not, yet they are usually more efficient for all three elements (Graham, 1984; Graham et al., 1987; Harry, 1982; Cooper et al., 1988). Studies of rye addition lines suggest that 6R contributes a little to efficiency for all three elements, perhaps by way of a root geometry feature, but the major genes are elsewhere. Manganese efficiency is located on 2R, a conclusion supported by the poor performance on manganese-deficient soils of cv. Coorong, an Armadillo-type triticale lacking 2R. By way of contrast, zinc efficiency in rye does not appear to be clear-cut from our studies of rye addition lines of wheat, and may be spread across four or five chromosomes: 2R, 3R, 7/4R and, to a lesser extent, as we've said, 5RL and 6R (Table 6, Graham, 1988a). Cakmak et al. (1997) have additionally linked Zn efficiency to 1R.

Zinc efficiency

Studies of addition lines have shown that Cu, Zn, and Mn efficiency in rye are independent traits, carried on different chromosomes (Graham, 1984). Copper and Mn efficiency in rye and Mn efficiency in barley appear to be controlled by single major genes (Graham, 1984; McCarthy et al., 1988), as is Fe efficiency in soybeans (Weiss, 1943). Boron and Mg efficiency in celery (Pope and Munger, 1953a,b) and B efficiency in tomato (Wall and Andrus, 1962) likewise appear to be controlled at a single locus (all reported to be dominant).

However, less is known of the genetics of Zn efficiency. Several loci on as many different chromosomes are involved in Zn efficiency in rye, and a few genes are involved in Zn efficiency in rice. The largest single screening exercise was done on 3,703 lines of paddy rice (Ponnamperuma, 1976; IRRI, 1979); 388 lines were judged to be tolerant and a similar range of responses was observed. Following diallel analysis, a recent report suggested that the genetic effects responsible for the Zn efficiency trait in rice are mostly additive, and to a lesser extent dominant (Majumder et al., 1990).

Soybean varieties differ in their response to Zn fertilizer (Rao et al., 1977; Rose et al., 1981; Saxena and Chandel, 1992). This may be a consequence of differential efficiency of Zn absorption; the distribution of F3 lines from the cross between Zn-efficient and Zn-inefficient genotypes (330 F3 lines tested) suggested that only a few genes control the Zn efficiency trait (Hartwig et al., 1991).

The various mechanisms of Zn efficiency in wheat are likely to be additive (as shown for rice, Majumder et al., 1990), which suggests that in a breeding program stepwise compounding of genetic information should be greatly emphasized (see Rengel and Jurkic, 1992). Such pyramiding into one locally adapted crop cultivar of a number of Zn efficiency mechanisms that are expressed at different levels of the plant organism (molecular, physiological, structural, or developmental; see Rengel, 1992) might

Picture 3. Varying degrees of tolerance to zinc deficiency in paired plots of barley lines growing in zinc-deficient soil, Horsham, 1998. One plot of each pair was treated with zinc fertilizer granules drilled with the seed and, later, a foliar spray. (Photo: J. Lewis.)

follow the approaches of Yeo and Flowers (1986). In such a breeding program, genotypes having genes controlling a particular mechanism of Zn efficiency may be very important even though they themselves may not show phenotypically high overall Zn efficiency. It would be advantageous to use local genotypes because this would expedite the development of cultivars with improved Zn efficiency without severely disrupting the broad adaptation already achieved (Picture 3).

Durati, a very sensitive durum wheat in the heavy black clay soils of New South Wales was also poor in our light sandy soils. It is therefore a valuable indicator line. Kamilaroi is a derivative of Durati (Durati x Leeds) which not only incorporates yield, quality and disease resistance from Leeds but also zinc efficiency on the heavy black earths of New South Wales. However, in South Australia on light sands, Kamilaroi appears worse than Durati for zinc efficiency. Zinc deficiency in the black earths is a complex phenomenon involving very high levels of native soil phosphorus and manganese that appear to aggravate the low zinc status. Thus zinc efficiency on these soils may not be so much a "foraging capacity of the roots" but a better discrimination for zinc over manganese and phosphate. In South Australia phosphorus and manganese are relatively low. We therefore recognize different types of zinc efficiency.

Besides diversity for yielding ability on zinc-deficient soils, there may be genetic control over zinc concentrations in tissue and grain (Graham et al., 1992). Excalibur and Warigal 5RL are generally superior to the other lines (30 tested in all) in zinc concentration in leaves and zinc uptake at tillering. However, Excalibur had low grain concentration, a condition apparently linked to its high

yield since grain zinc content (g/ha) was high. There is a distinct trend, as with grain nitrogen, for a lower grain zinc concentration with increasing yield (across genotypes). This is undesirable both because of lower seedling vigor when low zinc seed is used for resowing and because wheat is generally considered to be too low in zinc for adequate human nutrition when it forms a high proportion of the diet (Welch and House, 1983; Graham and Welch, 1996). However, Warigal stood out as having the highest grain zinc concentration and content under zinc-deficient conditions. It is highly likely that grain zinc concentrations could be improved by breeding. It should be noted, however, that grain zinc concentration responds dramatically to fertilization under these conditions.

Manganese efficiency

Remarkable diversity for manganese efficiency exists within wheat, especially in hexaploids and durums and this may be further supplemented, if warranted, with efficiency genes from rye.

Manganese efficiency in barley appears to be simply inherited, taking the evidence of the cross of Weeah (efficient) and Galleon (inefficient) (Graham, 1988b) and similar evidence of the cross between Weeah (efficient) and WI2585 (inefficient, McCarthy et al., 1988). This major gene has recently been mapped to 4H (see later section). However, another line WA73S276 that has common parentage with Weeah, has markedly more efficiency than Weeah, suggesting that other loci may be involved. Moreover, an important parent in the barley breeding program at the Waite Institute, CI3576 from Alexandria, is exceptionally susceptible to manganese deficiency, and so is a high percentage of its progeny. At this stage we are not sure whether this is some type

of semi-dominant manganese *in*efficiency trait, or an effect of tight linkage to another trait.

A low percentage of wheat cultivars also have exceptional sensitivity to manganese deficiency, the genetic basis of which is also unclear. However, recent studies (Khabaz-Saberi et al., 1998) has shown that Mn efficiency in durum wheat is contolled by two additive genes. It is likely that these are in homeologous positions in the two genomes, and from our experience with barley (single), rye (single) and durum wheat (2 genes), we can safely predict three major loci in bread wheat. Minor genes are almost certain to be involved in all species, but in the southern Australian breeding programs, much progress still needs to be made with these major genes.

The rankings for zinc and manganese efficiencies are somewhat inversely correlated (see Graham, 1990). Zinc-efficient Excalibur has poor manganese efficiency, and Bayonet, Millewa, and Takari are reasonably zinc efficient but acutely manganese inefficient. Others are reasonably efficient for both (Aroona, Machete) and some quite inefficient for both elements (Durati, Kamilaroi, Songlen, Gatcher). Durati
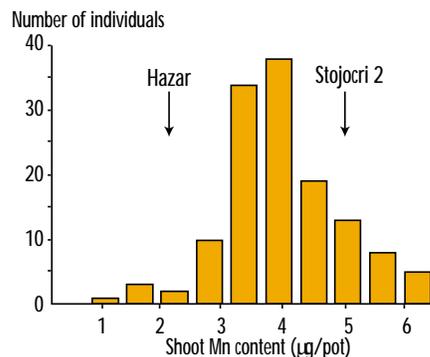

Figure 1. Frequency diagram of shoot manganese content (mg/pot) of F2 individuals from the cross of Stojocri 2 x Hazar durum wheats, when grown in manganese-deficient Wangary soil for 4 weeks.
Source: Khabaz-Saberi et al. (1998).

and Kamilaroi are poor for manganese, zinc, and copper. Indeed, most durums tested are poor for micronutrient efficiencies. Zinc efficiency in wheat, as earlier discussed for rye, appears also to be independent of copper efficiency.

Boron efficiency and toxicity tolerance

Both boron deficiency and boron toxicity are common nutritional imbalances of many crops, including wheat. Boron deficiency occurs mainly in highly leached soils of the humid zones while boron toxicity is more common in lower rainfall regions where limited leaching results in boron accumulating in the subsoil. Deficiency and toxicity affect different physiological processes and different tissues in wheat. Boron deficiency primarily affects pollen

Picture 4. Symptoms of boron toxicity on wheat and barley leaves. Barley is generally more sensitive and the symptoms more distinctive, making a sensitive variety like Stirling a good indicator line. (Photo: J. Coppi.)

development and pollination, while boron toxicity affects the growth of all tissues at all stages of development. A high level of genetic variation has been identified in wheat in response to both boron deficiency (Rerkasem and Jamjod, 1997) and boron toxicity (Moody et al., 1988). As the two imbalances are expressed at different stages of development contrasting screening methods have been developed to assist selection of required genotypes (Picture 4).

Identification of boron deficiency and boron toxicity can be undertaken, with varying degrees of success, by recognition of plant symptoms indicative of the disorders and by plant and soil analysis. As mentioned above, the major effect of boron deficiency of wheat is on development and function of pollen, thus sterility may occur in plants without foliar symptoms. The symptoms of boron toxicity of wheat consist of regions of chlorosis and necrosis developing from the tips and along the margins of the oldest leaves. While the symptoms for wheat are not readily distinguishable from symptoms of other stresses, boron toxicity symptoms of barley are very distinctive and consist of black spots developing within the necrotic lesions at the tips and margins of leaves. If boron toxicity is suspected, including a few plots of barley within a wheat trial will provide a rapid, low-cost means of diagnosing the problem.

Tissue analysis, using tissues such as youngest emerged leaf blades (YEBs), will give an indication of the boron status of the plant, and can be used to compare between genotypes in a high boron situation, but does not readily differentiate between efficient and inefficient genotypes under low boron supply. Boron concentrations in YEBs of less than 2 mg/kg might indicate

deficiency, while concentrations of greater than about 20 mg/kg in YEBs and 3 mg/kg in mature grain indicate a likelihood of boron toxicity.

Boron can be extracted from soil by a number of methods and the amount extracted will depend on the method and soil type. The most common methods used are hotwater and hot 0.01M $CaCl_2$ extractions. As the amounts extracted vary according to time, soil type and method, the absolute values only provide a rough guide to the amount of boron available to plants. A hot-water extractable boron concentration in soil of less than 0.5 mg/kg might indicate boron deficiency, while a concentration greater than 15-20 mg/kg is associated with toxicity. As high concentrations of boron in low rainfall environments are generally found in the sub-soil, the soil profile should be sampled to 1 m.

Additional methods of diagnosing the nutritional problem include application of the nutrient, in the case of a deficiency, and inclusion of probe genotypes in trials. Many Australian wheat varieties have been characterized for response to high concentrations of boron and several, including Halberd, Frame, and Spear that are tolerant, while Hartog is very sensitive. Appropriate probe genotypes for boron deficiency, identified in Thailand, include SW41 (inefficient) and Fang 60 (efficient).

Severe boron deficiency can result in complete sterility of an inefficient wheat genotype that produced healthy vegetative growth with a similar concentration of boron in the flag leaf and the ear as an efficient genotype with a high level of seed set. As no seedling or vegetative response has been identified that is correlated with seed set, it is necessary to select for efficiency at low boron supply during the reproductive stage.

Boron deficiency can be induced in sand culture in pots or in field trials. In both situations the response of the genotypes under test can be compared to an adequate boron control (application of 1 kg boron/ha, as borax), or to well characterized check genotypes (e.g., SW41 and Fang 60). The response is described by the grain set index, which is calculated as the number of grains in the primary and secondary florets of the 10 middle spikelets of the primary spike, expressed as a percentage of the potential (i.e., 20 grains). Restricting the number of grains to the primary and secondary florets, rather than counting total grain set, minimizes confounding effects such as moisture stress and other nutritional imbalances that influence the development of grain in the higher order florets.

Screening Techniques

Undoubtedly, the ideal is to understand the mechanisms involved and to select for the desired characters by means of their gene products. This may be phytosiderophore release as in the case of iron efficiency in wheat (Marschner et al., 1986), binding affinity in the membrane (Km), root geometry, or composition of simple root exudates that may control nutrient availability in the rhizosphere. If iron efficiency were a problem in wheat, it would appear a simple matter to select for greater ability of the roots to release deoxymugeneic acid under standard conditions of iron stress (as Marschner et al., 1986, have defined). The all-important advantage of this approach is that we are no longer dependent on measuring yield with all its potential for interactions (as the integration over time of gene expression on the limiting factor), but are measuring directly the intensity of expression of the efficiency alleles present in that genotype.

Field testing

Selection in terms of yield is always imprecise and fraught with difficulties: almost everything in the genome contributes to yield either directly or indirectly. If the selection pressure is great enough (that is, deficiency is severe and the primary limiting factor), then efficient genotypes will be selected based on yield, but the possibility that strong interactions will cloud selection is always there. Graham et al. (1992) reported inconsistent results between two sites for two lines of barley, one of which, Schooner, was able to respond to late (October) rains by virtue of its later maturity and, under warm soil conditions, it benefited from improved availability of native zinc in the soil. In this case, we believe the results from Lameroo, the other site, are more typical and reflect better the true zinc efficiency of the lines. Mid-season harvests help to support such interpretations.

Site selection. One of the most difficult problems with field studies is selecting an even site with a level of deficiency that will differentiate varieties for efficiency. Appropriate extraction techniques are unavailable, and soil analysis is poor in predicting trace element deficiencies. Field history is a valuable resource, but analyzing plant tissue taken from the wheat crop prior to the experiment is perhaps the most reliable tool for selecting a site. However, trace element deficiency is extremely dependent on environmental conditions (e.g., temperature, light intensity, and rainfall) and careful attention to tissue concentrations in previous crops may not ensure a deficiency in the experiment in a given year.

It is important that adequate levels of all other nutrients be applied as a basal fertilizer to all plots to avoid nutrient imbalances and interactions of other

nutrient deficiencies with genotype. One of the most difficult interactions to avoid has been that of boron toxicity in our zinc deficiency trials. Genotypes that are more susceptible to zinc deficiency exhibit greater symptoms of boron toxicity.

Two-level assessment. Our main approach to screening is to use plus-and-minus plot pairs to calibrate the performance of a genotype in deficient soil against its own potential with the limiting element supplied. Our primary efficiency index then becomes:

$$100 \cdot \frac{GY-}{GY+} \text{ or sometimes } \quad 100 \cdot \frac{veg\ Y-}{veg\ Y+}$$

where GY = grain yield, and veg Y = vegetative yield.

This is the parameter we call micronutrient use efficiency. However, we do not rely on this quotient alone, given that for various purposes, the lines with the highest GY- may be of interest when they have outstanding yield potential but still respond significantly to zinc (e.g., Excalibur; see Graham et al., 1992). Also of independent interest is GY+, the potential yield for a belt-and-braces approach, that is, a combination of nutrient and genotype that frequently outyields either alone (Graham, 1988a). We argue that $100 \cdot$ GY-/GY+ is probably the best basis for identifying parental material for a breeding program, whereas GY- may be of most immediate interest to producers when the problem is subsoil deficiency or topsoil drying, or when they are not willing to use micronutrients or are unaware of the deficiency.

It is obvious that a genotype can be characterized better by a yield response curve generated by increasing rates of fertilizer than by simple plus-and-minus treatments. However, in such studies only a few lines can be reasonably handled before the task becomes too large.

Particularly in field experiments, the increased area means increased spatial variability, a serious problem with micronutrients; this variability appears to be intrinsically greater than that for macronutrients (see Table 8, Graham, 1990). Moreover, the critical comparison between any given two rates (whether it be the plus-and-minus pair or any other pair) will be spread by randomization requirements over greater distances, with other treatments in between. Therefore, provided the site chosen has the degree of deficiency to be targeted in the breeding program, the paired-plot system has the advantage that the extreme proximity of the two treatments allows the most precise determination of $100 \cdot$ GY-/GY+ and that minimal size permits the comparison of the greatest number of lines.

This is the system we have used most in South Australia, but its justification depends on both the selection of relevant sites and on a perception of the number and nature of the genes involved. Paull (1990) found a number of genes involved in resistance to boron toxicity and proposed a model (to which Figure 2 is analogous for deficiencies)
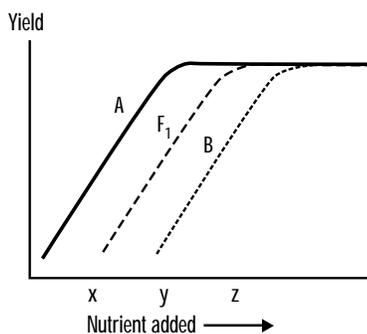

Yield

A
F_1
B

x   y   z

Nutrient added ——>

Figure 2. Model of the response to added micronutrient of two parents and their F_1 progeny showing how if screening them at a single level of stress, the genetic interpretation could be: at Z, A is dominant; at Y, partial dominance; at X, B is dominant (sensitive parent).
Source: On analogy with Paull (1990).

to account for the variable expression of dominance at different selection pressures. In his work, to select for/against all possible segregants in a tetragenic system, he selected at three different levels of stress.

In deficiency work, it is common to discuss the relative merits of the genotypes with yield-fertilizer rate responses like those in Figure 2. Genotype A is desirable because it reaches its potential at the lowest level of supply. But with micronutrients, it is common to add 10-100 times as much fertilizer as is absorbed by the crop, perhaps for several years (Fe, Mn excepted). Thus, the lowest rate of nutrient to achieve the yield potential is not determined by the paired-plot system; it can be argued that this is not critical and we need only a point safely on the yield plateau and a point at nil nutrient supply, provided the latter results in a meaningful degree of deficiency for the region targeted in the breeding program (for example, y in Figure 2). In this way we have justified our paired-plot approach in the past, and until such time as we have a better idea of the genes and mechanisms involved, it seems the most practical approach for our purposes.

Using the paired-plot system. A typical field experiment (using zinc as an example) currently consists of 36 genotypes x ± zinc x 4 replications, with border plots, laid out as a split-plot randomized block design. The experiment is sown with all main plots entered in pairs; one of each pair (chosen at random) receives zinc. Our seed and fertilizer drill uses a magazine system for delivering seed to a cone seeder, and zinc granules are delivered along with the seed via the magazine. The fertilizer box thus contains only basal nutrients (all other necessary nutrients, especially in our environment, nitrogen, phosphorus, sulphur, copper, manganese, molybdenum, cobalt) and does not need

to be changed. The zinc granules are commercial zinc oxysulphate (~ 30% zinc) used at several times the commercial rates (11-14 g per 4.5 m^2 plot) because its effectiveness is less than when coated on macronutrient granules (ammonium phosphate), the current commercial practice. The soil-applied zinc is supplemented with a foliar spray of zinc sulphate at tillering (equivalent to 200 g zinc/ha). (For manganese studies, manganese oxysulphate granules are used followed by one or two foliar sprays at 1 kg manganese/ha. For copper experiments, we have used copper sulphate granules and foliar sprays at 0.2 kg copper/ha).

Mid-season harvests are taken from 0.5 m^2 quadrants, partly to guard against loss of information due to end-of-season events (drought, hail, heavy late rains, sheep or cattle getting through the fence), and given that we escape the above, grain yield is measured by a small-plot harvester.

Because of the large spatial variation in the soils we study, advantage is taken of modern spatial statistical analyses, which have proved to be more efficient, that is, higher F, for the genotype x zinc interaction. Generally, a higher F is found in this analysis than from the simple split-plot randomized block factorial analysis.

The spatial ANOVA process is iterative and can identify non-treatment variation associated with the position of a plot or subplot in the field-plan array. We have identified variation due to the effect of tractor-wheel compaction on some plots and not on others, direction of sowing and harvest in relation to slope (up-down, left-right), prevailing wind (direction of head-bending), and depth of sowing. Removing variance of this type from the residual increases the significance of that due to treatment (Gilmour et al., 1997).

In addition to grain yield data analyses and the similar treatment of mid-season vegetative yields, a further index of considerable value is generated, after chemical analysis of the tissues and grain, by calculating the total uptake of zinc into vegetative growth and/or grain. The most efficient genotypes are so because they absorb more zinc and maintain higher zinc concentrations in vegetative tissues and often, but not always, in grain. The same is true for Cu and Mn. Uptake, being the product of yield x zinc concentration, frequently shows greater variation among genotypes than yield. (Compared with yield, variation in concentration is relatively small but usually significant). Uptake reflects in some measure the expression of the nutrient use efficiency trait integrated over time from sowing to harvest (Picture 5).

Single-level assessment. Single-level assessment is conducted without control plots supplied with the limiting nutrient, which are replaced for purpose of interpretation by plots of a check genotype. A typical design that proved useful in our manganese program (Graham et al., 1983) involved 196 plots and only two complete replications of the 72 barley lines tested. The remaining 52 plots were check plots of one cultivar located every fourth plot in a regular array.

The purpose of this experiment was to identify genotypes as nutrient-efficient as the check cultivar (or better) by using an acutely manganese-deficient site. The results can be analyzed by spatial analysis techniques (ASREML) and by comparing test plot yield to the check genotype yield surface for the site generated from the array of check plot yields. More simply, plot yields can be compared to the arithmetic mean yield of adjacent check plots. The check plot yield array has proved highly efficient at defining site variability and

Picture 5. Paired-plot screening of wheat breeding material for zinc efficiency, Horsham, 1998-99 (Photo J. Lewis). Plus and minus zinc plots of the one entry are sown side by side, with zinc applied to one plot chosen at random. The trial consists of 30 lines sown in four replications in a randomized complete block design, with the data subjected later to spatial ANOVA (Graham et al., 1992).

Picture 6. Single-plot screening of barley breeding material for manganese efficiency, Wangary, 1981. No Mn fertilizer was used but a Mn-efficient check was sown every fourth column throughout, with single plots of 72 test lines sown in between (Graham et al., 1983). Two replicates. In the column to the left of center, the four plots are (starting from the front) efficient, inefficient, inefficient, efficient. A column of an efficient check runs the length of these plots on the left; parts of three other check plot columns can be seen to the far left and right. (Photo: R. Graham.)

fertility trends. Consequently, where a wide range of performance is expected among the entries, efficient selection may be achieved with entries appearing only twice in the matrix. Quite a number of borderline genotypes may not be convincingly placed in either the efficient or inefficient group, but usually this is not important. The efficiency of selection in this simple design was underlined by the meaningful genetic relationships discovered in both the efficient and inefficient groups (Graham et al., 1983) (Picture 6).

Measurements. In field work measurements of grain yield should be supported by observations that assess efficiency at seedling and mid-season growth stages. Atypical weather can confound measurements at a single stage of growth (such as late rains that favor late-maturing genotypes). Chlorosis, vigor, and delayed maturity (heading date) can be scored by eye. However, quadrat sampling (or 1 m of row) at mid-season can quantify vegetative yield and, equally important, provide plant material for analysis. Total uptake (dry matter yield x concentration) is a valuable index of nutrient efficiency that integrates root system vigor and efficiency with shoot requirement. Genotypic differences in critical concentration are recognized (Ulrich and Ohki, 1966) but in our experience are relatively small compared with the total uptake for defining nutrient efficiency.

Collecting and analyzing plant samples. A reliable measurement in assessing our field experiments has been to analyze whole plants and youngest expanded leaf blades (YEBs) by inductively coupled plasma (ICP) spectroscopy (Zarcinas et al., 1987). It is important that plant material be as free from contamination (for example, dust, soil particles, galvanized products such as gates and tools, cigarettes, and some paper bags) as possible. We recommend the use of

plastic gloves for handling both YEB and whole plant collection. When collecting whole plants, plants are cut approximately 1 cm above ground level to minimize contamination with soil. They are then stored in suitable paper bags and dried overnight at 80 °C.

In our experience manganese efficiency may vary 10-42%, with grain yields of -Mn plots from 0.1 to 0.8 t/ha. Durati, Takari, and Millewa are so inefficient and the +Mn fertilizer treatment (soil + foliar) so ineffective that the +Mn plots were still deficient (young leaves 12 mg/ kg Mn) and perhaps yielded barely half their potential (Graham, 1990). Although not a great problem, this results in higher efficiency indices than the inefficient lines warrant, making the extent of diversity for this character actually greater than measured.

At the manganese-deficient site, the resistance of Aroona, Machete, and Millewa to the take-all fungus was in line with their manganese efficiency at the vegetative stage (Graham, 1990; Pedler, 1994). Machete improved its ranking from 19th at tillering to 7th at grain harvest, while Gj*Wq faded from 2nd to 21st. While only a few lines changed ranking markedly through the season, this meant that selecting the top five at tillering would have netted only three of the top five at maturity, a point in favor of selection in the field (Picture 7).

Screening in controlled environments

Soil cultures. Using soil in pots requires less effort to set up and maintain than solution cultures, and the work is less labor-intensive than field sites. The same soil requirements apply to screening in field or in pots, but in pot work the experimenter obtains, by thorough mixing, a uniform soil for each genotype tested, albeit in a quite atypical environment. The latter is particularly important. Nutrient stresses are frequently associated with low soil temperatures, and uncontrolled soil temperatures in the glasshouse can be extremely unrealistic. For example, it is often difficult to produce manganese deficiency in glasshouse conditions, even with soil that is severely deficient in the field, and low temperature baths or

Picture 7. Paired-plot screening of parents and breeding lines for manganese efficiency, Wangary, 1987. Manganese was delivered as Mn oxysulfate granules with the seed and 1-2 foliar sprays of Mn sulfate solution applied mid-season as required. (Photo: R. Graham.)

controlled-environment rooms are necessary to keep temperatures below 15 ºC. The results of Fox (1978) are an excellent example of the genotype x climate x nutrient interaction preventing the correct interpretation of nutrient efficiency tests.

The size of pots has also been shown to be an important consideration when screening for nutrient efficiency. While it is tempting to use small pots in the growth chamber to allow the screening of as many lines as possible, it has been shown that for manganese efficiency, pot capacity should not be less than 0.5 kg soil for two wheat seedlings grown for 28 days. Pot size should roughly double for each week of growth beyond 28 days (Huang et al., 1996).

We have done considerable work in pots, usually vegetative growth studies, but we have occasionally found the rankings quite disparate with field-based grain-yield rankings, which suggests mid-season or later effects can be important. Examples found in Marcar and Graham (1987), Rerkasem et al. (1990) and in our recent screening for zinc efficiency in wheat (R.D. Graham et al., unpublished) suggest that field screening is important. In screening for manganese efficiency in barley, only one of two major gene loci contributing half of the trait each could be screened for in pots. The other, it seems, much be screened for in the field.

Screening for copper, zinc, and manganese efficiency in pots seems to be satisfactory (Graham and Pearce, 1979; Grewal and Graham, 1997; Rengel and Graham, 1995a; Huang et al., 1996), except as mentioned above. When screening for manganese efficiency in pots, both the storage, temperature, and moisture effects on manganese availability and the screening technique (Longnecker et al., 1991; Webb et al., 1993a; Huang et al., 1996) are critical.

The mechanism of manganese efficiency has proved quite elusive, but empirical screening has been effective enough to lead to the development of a molecular marker for the major gene involved (see later).

Screening for boron toxicity and deficiency in pots has been effective. High boron supply results in chlorotic and necrotic lesions at the tips of the older leaves, reduced plant vigor, restricted tillering, poor root elongation, and elevated concentrations of boron in all plant tissues. Tolerant genotypes maintain lower boron concentrations in tissues, develop less severe symptoms of toxicity, and produce more vigorous shoot and root growth than sensitive genotypes. These differences in response have been used to develop efficient screening systems that have assisted in breeding boron tolerant wheat varieties (Moody et al., 1988) and enabled the identification of major genes controlling boron tolerance and their locations on chromosomes 4A and 7B (Paull, 1990; Chantachume et al., 1994).

There is a strong correlation between the response of wheat to high boron concentrations during seedling growth and at later stages of development. This has enabled the development of several rapid seedling assays to identify boron tolerant genotypes. One method involves growing seedlings in a glasshouse. Additional boron in the form of boric acid is uniformly mixed through fertile clay-loam soil to give an extractable boron concentration in the range of 50-80 mg/kg. Plants are watered well for several weeks, until they are established, and then less frequently. During this second phase, roots of the more tolerant plants will be able to extract moisture from deeper in the soil profile, and these plants will continue to grow. Roots of the sensitive genotypes do not grow in

the high boron soil, and the plants appear stunted. Tolerant genotypes also develop less severe symptoms of boron toxicity.

Solution cultures. Simple solution cultures can rarely be used to select nutrient efficiency factors that operate on some feature of the root-soil interface. Efficiency factors operating within the root surface may be screened for in solution: characteristics of the absorption isotherm, xylem loading, short- and long-distance translocation, and efficiency of nutrient utilization (for example, carbon fixed per unit of nutrient absorbed). Such micronutrient efficiency factors operating internally (e.g., boron translocation in tomato; Wall and Andrus, 1962) are also dealt with by soil techniques.

Special adaptations that operate in soil may be successfully observed in solution cultures under certain conditions. Brown and Ambler (1973) used strong iron chelates in solution to study the reducing power of the root, since the reduction Fe^{3+} to Fe^{2+} was necessary to break the ligand-Fe^{3+} bond and free ionic iron for absorption. Clark et al. (1982) used solutions modified with high phosphate, nitrate, and calcium carbonate to induce iron stress in susceptible sorghum genotypes.

Iron and manganese efficiencies may be studied in solutions containing suspensions of insoluble iron hydroxide or manganese dioxide, both of which require reduction for dissolution; this process is promoted by proton extrusion (see Brown, 1978; Romheld and Marschner, 1981; Uren, 1982). These insoluble higher oxides/hydroxides may also be precipitated on chromatography paper (Uren, 1982) and roots made to grow along the wet paper surface, a system that is a compromise between soil and solution culture. Effective reduction and dissolution of the dark-

colored oxide by the root is detected by a white depletion zone on either side of the efficient root. For effective screening of genotypes, differences must be quantified, requiring good quality control over the precipitation process and test conditions.

Higher iron concentrations are on occasions found in chlorotic tissues than in green tissues (Brown, 1956), and up to 100 times more iron may be found in roots than in shoots of iron-deficient plants (Brown, 1978). These observations show that there are efficiency mechanisms operating within the plants that may be tested for in solution cultures.

Flowing culture systems add another dimension to solution culture approaches. With this technique, it is possible to define for each genotype the lowest solution-phase concentration of an element that can sustain maximal growth rates. This is clearly a genetically controlled character (Asher, 1981). Such systems, while too expensive for routine screening, provide information about physiological mechanisms to aid in developing rapid screening tests.

Solution techniques have been most widely and successfully used in screening for tolerance to mineral toxicities and in elucidating the mechanisms and genetics involved. Screening and selecting for aluminum tolerance, for example, has been efficiently carried out in solution cultures (Furlani et al., 1982; Foy et al., 1978; Reid, 1976).

The relatively new technique of chelate-buffered nutrient solution culture has potential in screening for micronutrient traits (Webb et al., 1993b; Huang et al., 1994a,b; Rengel and Graham, 1996). However, caution and more development are needed in terms of activities of ions in solution and interpretation of results.

Boron tolerance. The effect of boron on root growth has been utilized to develop a rapid, objective assay to identify genotypes tolerant to boron toxicity (Chantachume et al., 1994). Seeds are imbibed in petri dishes at 2-4 ℃ for 2 days, then at 18-20 ℃ for 1 day. Large, rectangular filter papers or absorbent paper towels are soaked in a solution of boric acid (concentration in the range 5-10 µM), 0.0025 µM zinc sulphate, 0.5 µM calcium nitrate, and basal nutrients, and then allowed to drain for 1 min. The seeds are placed in a row across the top third of the paper, with the embryos facing the bottom. The towels are rolled up into a cylinder, enclosed in aluminum foil and stood on end with the embryos facing down and stored at 15 ℃ for 12 days. The towels are then unrolled and the lengths of the longest roots are measured. Tolerant genotypes develop longer roots than sensitive genotypes. Tolerant and sensitive controls should be included in each filter paper, and reference should be made to a control filter paper without boron to compare relative root lengths.

In a modification of this method by Campbell et al. (1998), seeds were sown on a fine mesh over a "lunchbox" containing the boron solution, with aeration. Tolerant and sensitive genotypes are again distinguished on the basis of root growth. This method has the advantage of being less labor-intensive and less expensive because filter paper and aluminum foil are not required; however, correlations with other screens may not be quite as good. Nevertheless, the ranking of well characterized genotypes is consistent among the alternative screening methods and with the concentration of boron in shoots and grain when grown in fields with high concentrations in the sub-soil. The root length screen can be combined with a measure of coleoptile length where this is important (Picture 8).

Importance of seed quality in screening for micronutrient efficiency

Seed with high mineral content has been shown to be associated with early seedling vigor; this early advantage due to nutrient content of seed may still be observed at maturity as increased grain yield, larger seed size, and more grains per plant (Longnecker et al., 1991; Rengel and Graham, 1995a, b), even where nutrient supply is non-limiting for plant growth. The nutrient content of seed is dependent on soil type, nutrient availability, species, and to a lesser extent, variety and season. Longnecker and Uren (1990) showed for both barley and white lupin that seasonal effects had less influence on seed Mn content than choice of site.

It has already been demonstrated that there is genetic potential for wheat genotypes to grow at sub-optimal levels

Picture 8. Rapid screening for boron tolerance on wheat seedlings in solution culture.[†] (Photo: ETU, University of Adelaide.)

[†]   Boron concentration: 100 mg/liter, as boric acid (Campbell et al., 1998).

of micronutrient supply. Since the addition of micronutrients has in many cases decreased the incidence of diseases in wheat plants (Graham, 1983; Graham and Webb, 1991) and since differences in nutrient content of seed are also under genetic control (Moussavi-Nik, 1997), there exists a large overlap of factors that may confound the performance of genotypes.

Nutrient content of seed has also been shown to affect the susceptibility of wheat to diseases, for example, McCay-Buis et al. (1995) demonstrated that wheat plants grown from seed with higher manganese content (1.83-2.28 μg seed^{-1}) were more vigorous, had less take all, and produced more grain than plants grown from seed with lower manganese content (1.26-1.86 μg seed^{-1}). J.L. Cooke (thesis in preparation) reported less damage due to *Rhizoctonia* in wheat plants that had been grown from seed

with high zinc content (0.66-0.74 μg seed^{-1}) than in wheat plants grown from seed with low zinc content (0.24-0.26 μg seed^{-1}) (Picture 9).

For durum wheat cultivars differing in manganese efficiency, Khabaz-Saberi et al. (2000) developed a correlation between seed manganese content and the amount of added Mn required in Wangary soil to assess the Mn efficiency in a pot bioassay. This correlation differed for the manganese-efficient durum wheat cultivar Stojocri 2 compared to the manganese-inefficient Hazar.

When comparing genotypes for dry matter production, grain yield, grain quality, disease resistance, and nutrient efficiency, it is important that the quality of seed sown be similar for all genotypes to avoid confounding effects. However, seed size and nutrient distribution within the seed has been shown to be under genetic control (Moussavi-Nik, 1997). This means that seed of genotypes collected from the same site (soil type) and season may differ notably in nutrient content. In an extensive series of experiments involving 11 wheat genotypes, seed for each genotype was selected from eight localities in South Australia and tested at eight locations over two contrasting climates.

Moussavi-Nik (1997) demonstrated that genotype effects accounted for the largest portion of treatment variance, while seed source effects accounted for from almost none to 44% of the treatment variation and were significant in 9 of 16 of the experiments conducted. Generally the interaction between genotype and seed source contributed less to treatment variation than seed source main effects. Significant seed source or genotype x seed source effects occurred over the whole range of environments tested, from the highest to the lowest yielding. The seed selected from all locations had adequate nutrient content for all elements investigated; the eight sites used for assessing the effects of seed source were all adequate for trace elements. In spite of this, there were four significant associations of increasing zinc content of seed resulting in increased grain yield and one association of sodium seed content decreasing grain yield.

A Breeder's Approach

Knowledge of the mechanisms and inheritance of trace element efficiency simplifies the breeding and selection of improved lines because the choice of parents and the screening methods can be more targeted and objective. However, lack of this information must not deter a breeder when trace element deficiencies are known problems in his target area. Significant advances can be made while the genetics is still being investigated.

Potential parents for trace element efficiency can be sourced from the literature, by reputation and, particularly, from cultivars that continue to be grown by farmers in trace element deficient areas, despite the fact that crop evaluation trials indicate that these cultivars have been superseded by new, higher yielding cultivars.

Picture 9. Seedling vigor of zinc-inefficient Gatcher wheat growing in zinc-deficient soil, showing the importance of seed zinc content to establishment (Rengel and Graham, 1995a).

Since trace element efficiency is only one objective in an overall breeding program, it should be incorporated into the breeding effort and fitted in with strategies and methodologies used for selecting for disease resistance, yield, adaptation, and quality. Once potential parents have been chosen and appropriate crosses made, an important part of the strategy is to set up nurseries which with plant or line selection will enhance the frequency of genes in the breeding population for trace element efficiency.

A critical consideration in such nurseries is seed source, given that the trace element content of seeds can greatly affect early plant vigor and even final grain yield (previous section). Seed low in Mn content, harvested from Mn deficient sites, is best for screening for Mn efficiency; however, such seed may not be available in a normal breeding program. It is important therefore to use seed as close to the same Mn content as practical, and as low as possible (probably not seed produced on a research station, where soil fertility tends to be higher than on farms). All comparisons should be made using seed from the same original nursery. To reduce the effects of seed Mn content on selection, the Mn selection nurseries should be grown in soils with an acute deficiency. In South Australia selection sites for Mn efficiency have a highly calcareous soil in which Mn is tightly bound and poorly available to inefficient plants.

The flow chart in Figure 4 details how selecting for Mn efficiency is incorporated into the wheat breeding program in South Australia, as a model for any micronutrient efficiency trait. Note that it is no different from incorporating selection for resistance to a root disease such as cereal cyst nematode, where much of the screening is done in specially chosen field sites.

Early generation screening

In early generations populations expected to segregate for Mn efficiency are grown in spaced-plant nurseries in soil known to be acutely deficient in Mn. Bulk F2 and/or F3 populations are planted in long rows using a precision seeder with 10 cm between seeds in a row. Rows are 30 cm apart. This arrangement allows for easy observation of single plants.

In these soils even small changes in available manganese can cause large differences in plant growth response. To be able to get some indication of this and to take it into account when selecting in the nursery, indicator rows are sown at regular intervals throughout the nursery, usually as a pair every 7th and 8th row. Cultivar Yarralinka, recognized as being very manganese efficient, and Millewa, a very inefficient variety, are planted as paired rows. Contrasting growth between these two control cultivars indicates very deficient areas where meaningful selection of single plants from adjacent rows can be carried out. Where the growth between the two rows is similar, the Mn deficiency is not such a limiting factor or there are other more limiting factors and selection for Mn efficiency will not be effective.

Long rows are better than short plots because they will traverse across the variability that occurs and there will nearly always be sections of each row where selection can be practiced. Single plants or single heads are selected and these are treated in a routine way in the breeding program to select for other attributes. Whether the selections from each cross are bulked or whether they are kept as separate progeny, the population resulting from this nursery is enhanced for Mn efficiency genes. This selection process can be repeated.

Later generation screening

In later generations lines should be evaluated for yield in locations, soils, and farmers' fields where Mn problems are expected as part of the range of sites used in assessing adaptation. Thus Mn efficiency will be measured as part of the region's overall genotype x environment effects. In areas where there are strong abiotic factors limiting yield, the breeder should be testing and selecting in the presence of the stress and selecting for stress tolerance rather than for yield *per se*. Such sites are not usually found on research stations that have been chosen and/or managed for yield potential and that are free of abiotic stresses.

To get a measure of Mn deficiency directly (which the breeder needs to know to do further selection and crossing), lines are assessed in a known Mn-deficient site in replicated split-plot experiments, with genotypes as the main plots and different levels of Mn fertilizer as the subplots. Fertilizers (P and N) are applied at normal recommendations, and there are nil and applied Mn subplots. On Mn fertilized subplots an initial dose is applied to the soil at seeding as manganese oxysulphate; further applications are made as foliar sprays of manganese sulphate at tillering and again during stem elongation if needed.

Control cultivars include Yarralinka and Millewa, as well as cultivars being grown commercially in the area. The performance of this pair indicates the responsiveness and success of the experiment. Yield data are spatially analyzed using ASREML software, and mean yields with and without applied Mn are plotted against each other (Figure 3).
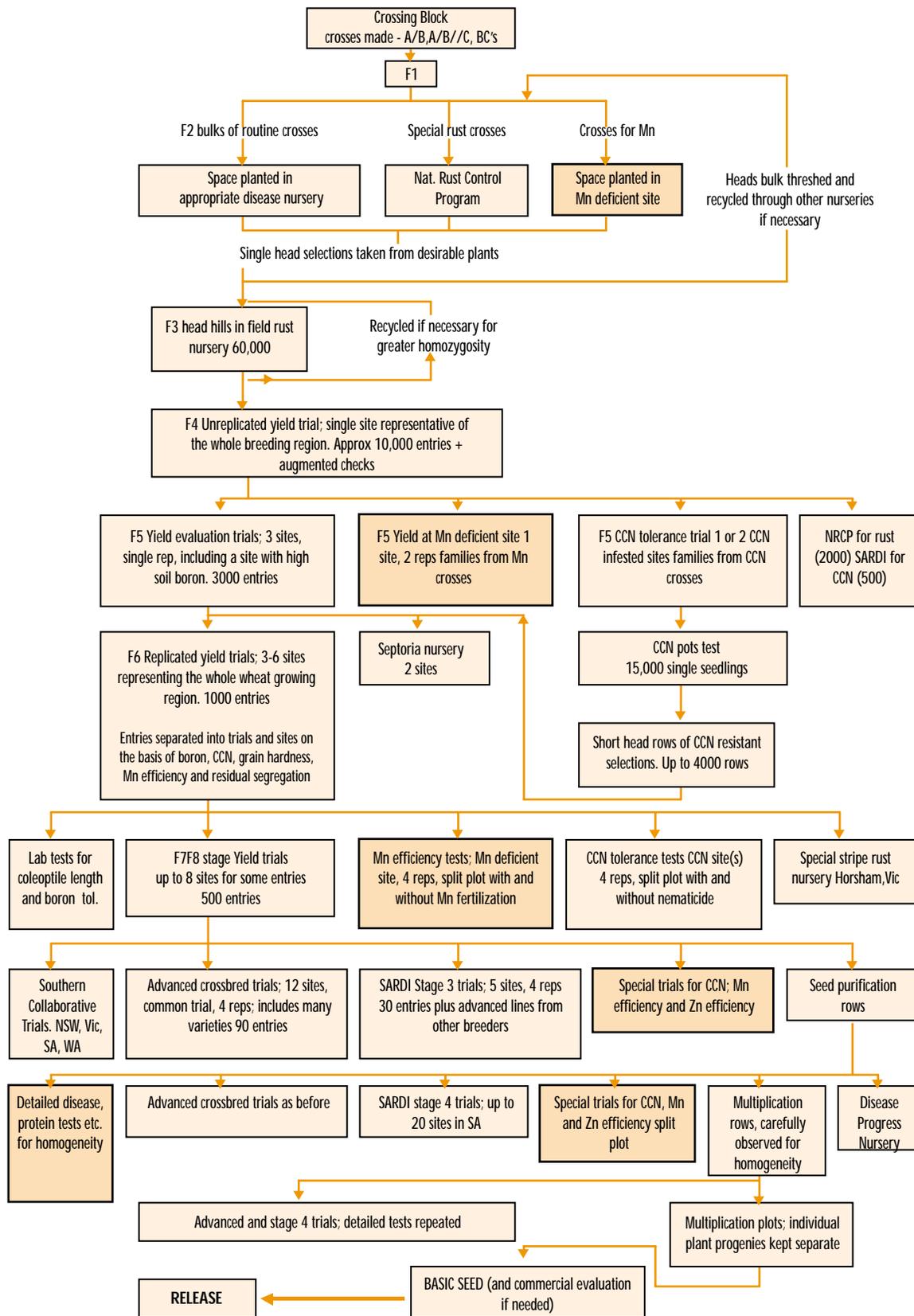
Figure 4. The Roseworthy modified pedigree wheat breeding program, describing the integration of breeding for cereal cyst nematode (CCN) resistance and manganese efficiency within the program. Heavy boxes mark specific activities for Mn efficiency.
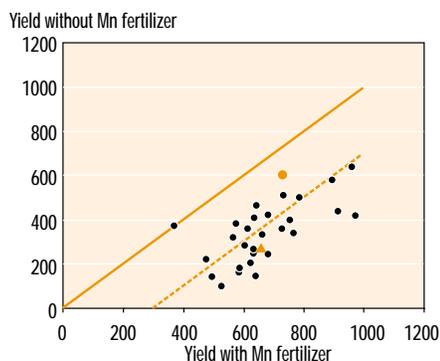
Figure 3. The regression of yield of genotypes without manganese fertilizer on yield with Mn fertilizer, in a field trial on Mn-deficient calcareous sand at Marion Bay.

Use of Molecular Markers in Screening for Micronutrient Efficiency

Molecular markers simplify selection for micronutrient efficient genotypes

Selection of micronutrient-efficient lines from segregating populations is desirable but difficult to do in either field trials or glasshouse and growth-chamber pot bio-assays. The primary reason for this is the requirement for specific growing conditions to maximize genotype differentiation. This is not always attainable in the field and, consequently, screening in field trials is expensive and may not be successful in some seasons such as droughts. Even in the glasshouse or growth chamber where day length, temperature and lighting can be controlled, variation in expression of the trait can be affected by other factors, such as the nutrient status of the seed (Uren et al., 1988) and the length of time and conditions in which the soil used to grow the plants was stored (Webb et al., 1993a).

Another difficulty to overcome is the problem of differentiating heterozygotes, even those which may be intermediate in phenotype, from homozygotes. Our studies have shown that this is not easily done, even where the trait is primarily controlled by a single gene that can express (depending on environment) as dominant, semi-dominant, or recessive, as appears to be the case for manganese efficiency in barley (Pallotta et al., 1999). Separating genotypes in situations where several genes control the trait, as is likely to be the case for hexaploid wheat, is exceedingly difficult and requires extensive progeny testing.

Identification of micronutrient efficient genotypes in early generations of crosses is virtually impossible since each individual plant represents a different genotype. In addition, genotypes should be tested at both low and sufficient micronutrient availability to compensate for genetic variation in plant response due to segregation of genes independent of the nutrient effect.

Where traits are difficult to reliably assay, as are micronutrient efficiency traits, there is a strong case for the use of marker assisted selection (MAS). Our group has identified several RFLPs (restriction fragment length polymorphisms) closely linked to a major gene (*Mel* 1) controlling manganese efficiency in barley (Pallotta et al., 1999); these are currently being used for early generation selection and to facilitate rapid backcrossing of the trait into elite breeding lines.

A second major locus in barley, *Mel* 2, has just been mapped. Work is under way to genetically characterize and map the manganese efficiency trait in the durum wheat cultivar 'Stojocri.' Results indicate the trait is semi-dominant, as was found in our studies on barley and segregation in an F_2 population fitting a two-gene model (Khabaz-Saberi et al., 1998). Genetic variation for manganese and zinc efficiency has been reported in hexaploid wheats (Graham, 1988b; Graham et al., 1992; Grewal and Graham, 1997). Efficient wheats have a yield advantage when grown in environments where zinc or manganese is limiting. Mapping genes controlling these traits in both species would assist the efficient breeding for these traits.

Marker-assisted selection is especially useful in mainstream breeding programs to accelerate backcrossing. The trait of interest can be followed in successive backcross generations by MAS with no need for phenotyping, given that the overall performance of the recurrent parent is usually well known. Two breeding schemes are shown in Figures 5 and 6, for the backcross and intercross programs. Marker-assisted selection can substantially reduce the time required for variety development.
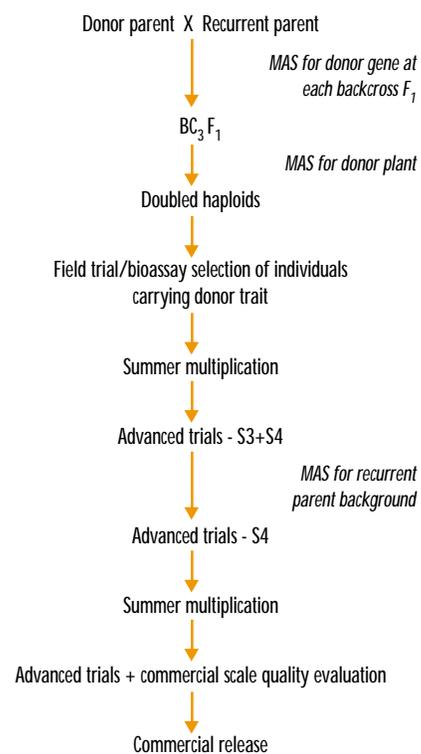

Figure 5. The flow-chart indicates where MAS (marker-assisted selection) can be used in a backcrossing program. The scheme facilitates the rapid incorporation of one or more desired traits into an elite background.

Doubled haploids aid in screening for micronutrient-efficient cultivars

Marker-assisted selection is especially useful when combined with doubled haploid (DH) technology. If molecular marker technology is unavailable, the use of DH populations provides an option for improved selection of micronutrient-efficient lines. Doubled haploid populations make early generation screening by traditional methods more accurate because all progeny of each DH line are genetically identical and true-breeding, enabling replication of tests, which is desirable.
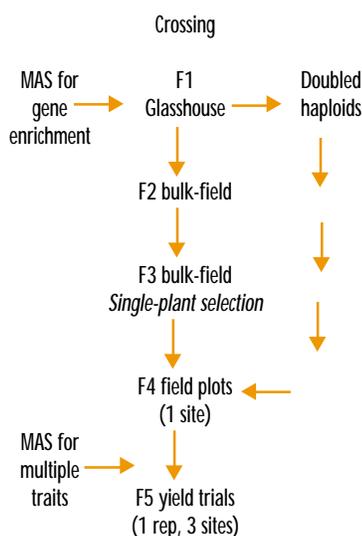

Figure 6. The flow chart indicates where marker-assisted selection (MAS) can be used in an intercross breeding program. MAS would be used where either a backcross or a topcross is involved.

However, precautions need to be taken to reduce the variations in kernel size, the position of the kernel in the spike, and the conditions under which different spikes of the same plant mature. It is necessary to carefully control the ripening conditions of the primary DH plants and to select for uniformity of kernel size.

Doubled haploid populations offer other advantages over recombinant inbred populations when molecular markers are available, particularly if the molecular marker for a trait is a co-dominant marker because the need for progeny testing is eliminated. Doubled haploids in combination with molecular markers provide a powerful tool for early generation screening of micronutrient-efficient genotypes.

Molecular marker-assisted selection can be used to pre-screen the F_2 plants used for DH production and increase the proportion of DH lines carrying the desirable gene(s). This is illustrated in Figure 7 for a single gene for micronutrient uptake efficiency where F_2 putative donor plants are tested for the presence of molecular markers closely linked to the efficiency gene. Selection of F_2 donor plants may be restricted to homozygotes only (homozygote selection) or may also include heterozygotes (allele enrichment). A comparison between homozygote donor

plant selection, allele enrichment, and no MAS is made in Table 1, which tabulates the proportion of F_2s and F_2-derived DHs expected when selecting for independently segregating genes at 1, 2, 5, or 10 gene loci.

It can be seen from Table 1 that with homozygote selection, 100% of the DHs produced will possess the desired alleles irrespective of the number of gene loci under selection. However, the proportion of donor plant F_2s which are homozygous at each locus decreases exponentially with increasing number of loci so that the probability of selecting five genes homozygous for the desired allele would be 9.77 x 10^{-4}, or less than 1/1,000. This is actually less favorable than using no donor plant selection at all, where 3.13 x 10^{-2}, or around 3% of the DHs derived from unselected F_2s, would be expected to carry all five favorable alleles.

With allele enrichment, the proportion of F_2 donors carrying at least one favorable allele from each of five genes would be 24%. It would be expected that 14% of DHs produced from these selected donors would carry all five desirable able alleles. That is, approximately 1 in 4 F_2 plants can be selected as donors for DH production and among these 14% will carry the desirable allele at each of the five gene loci. Allele enrichment is clearly a more efficient procedure for combining DH technology and MAS than homozygote selection, particularly when larger numbers of genes are being screened.
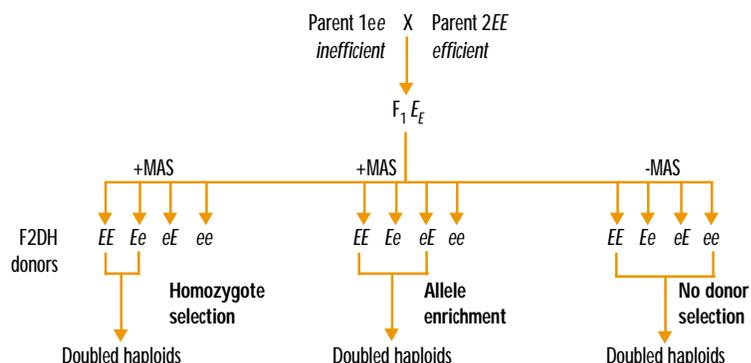
Doubled haploids for the elucidation of molecular markers

Doubled haploids are also very useful for genetic and mapping studies of micronutrient efficiency traits, as all lines in a DH population represent the result of a single meiotic event, whereas

Figure 7. Selection of F_2 donor plants with and without marker-assisted selection (MAS).

F_2s are the summation of a male plus a female meiosis. The DH lines therefore reflect the results of recombination precisely without the necessity for statistical inference.

In both wheat and barley DHs are being widely used for mapping many different genetic traits. In addition to the barley manganese efficiency genes described above, a DH population derived from a cross between two zinc-efficient bread wheat lines, cultivar 'Trident' and breeding line 88ZWK043, exhibited transgressive segregation in a pot bioassay for zinc efficiency; segregation data suggests several genes control the trait in this cross.

Conclusions

Genotypic variation exists for tolerance to practically every abiotic stress in every crop investigated. The level of tolerance available in elite germplasm is agronomically valuable and justifies breeding efforts in most cases, not just

those for which there are no other successful agronomic solutions. Inheritance varies from simple to quantitative, and both genotype and soil type, as well as climate and season, affect the expression of a trait. Often these traits are still manageable in a breeding program, and the $G \times E$ interaction is not prohibitive of the effort.

Screening for deficiency tolerance traits is, however, much more difficult than for toxicity tolerance traits, owing to quite sophisticated inducible systems that respond to deficiency in the plant by mobilizing nutrient bound in the soil that is not otherwise available. Strong expression of these systems is the objective. However, in view of the difficulty in developing fast methods for screening for efficiency (such as seedling selection in pots) or their limited ability to reflect field screening, molecular marker assisted selection is considered important for success in practical breeding programs, and a number of major gene loci already identified make this possible.

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Table 1. Selection strategies for combining doubled haploid (DH) technology with marker assisted selection. Probability of obtaining F_2 donor plants homozygous or heterozygous for the desired allele(s) at n loci and the proportion of DHs expected to be homozygous for these alleles when only homozygotes are used as DH donor plants (homozygote selection), when either homozygotes or heterozygotes are used as DH donor plants (allele enrichment), or when selection is not practiced on donor plants.

| No. of gene loci | Homozygote selection | | Allele enrichment | | No donor selection |
	F_2s homozygous for desired allele at each locus	DHs homozygous for desired allele at each locus	F_2s homo- or hetero-zygous for desired allele at each locus	DHs homozygous for desired allele at each locus	DHs homozygous for desired allele at each locus
	$(1/4)^n$	$(1)^n$	$(3/4)^n$	$(2/3)^n$	$(1/2)^n$
1	0.25	1	0.75	0.67	0.50
2	6.25×10^{-2}	1	0.56	0.45	0.25
5	9.77×10^{-4}	1	0.24	0.14	3.13×10^{-2}
10	9.54×10^{-7}	1	5.6×10^{-2}	1.7×10^{-2}	9.77×10^{-4}

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