

CHAPTER THREE

MATERIAL AND METHODS

3.1 Plant materials

Thirty-six barley lines (referred here after as genotypes) (Table 1) representing different maturity and disease resistance levels were selected. These lines were part of 172 barley genotypes of wide eco-geographic origin initially obtained from Ethiopian gene bank, based on their reactions to diseases and days to heading. The preliminary screening was done at Debre Birhan research site in 2004 growing season.

Table 1: The list of genotypes used in the study

Line	Acc.#	Code	Maturity and resistance level	Admin. Region	District	Altitude	Line	Acc.#	Code	Maturity and resistance level	Admin. Region	District	Altitude
18	3235	1	ER	Gondar	Gzur	2350	12	3235	23	ES	Gondar	Gzur	2350
5	3244	2	ER	Gondar	Alefa	1790	8	3235	24	ES	Gondar	Gzur	2350
2	219910	3	ER	Tigray	Adi	1870	17	3244	25	ES	Gondar	Alefa	1790
3	219910	4	ER	Tigray	Adi	1870	23	3244	26	ES	Gondar	Alefa	1790
15	1706	5	MR	Arsi	Bilbil	2500	12	325	27	ES	Tigray	Wukro	1900
3	1791	6	MR	Shewa	kewet	2500	5	3235	28	ES	Gond	Gzur	2350
2	3295	7	MR	Bale	Ada	2450	5	208733	29	ES	Welega	Sey	1880
9	64166	8	MR	Gamu	Che	3000	9	1622	30	MS	Shewa	Werena	2120
17	64210	9	MR	Bale	Dinsh	3400	3	1753	31	MS	Shewa	Amb	3340
14	3302	10	MR	Arsi	kofele	2640	2	4300	32	MS	Harar	Kurfa	2630
14	64068	11	MR	Gondar	Lay	3160	18	263	33	MS	Tigray	Hager	2600
1	1621	12	LR	Shewa	Werena	2480	1	3284	34	MS	Bale	Ginir	2960
16	1622	13	LR	Shewa	Werena	1622	7	367	35	MS	Tigray	Adig	2500
4	1791	14	LR	Shewa	Kewet	2500	19	407	36	MS	Tigray	Bole	3000
1	3232	15	LR	Gojam	Banja	2460	1	1651	19	LS	Shewa	Werena	2120
20	3304	16	LR	Arsi	kofele	2570	12	244	20	LS	Tigray	Shinko	2800
16	3978	17	LR	Welo	Des	2500	4	3304	21	LS	Arsi	kofele	2570
12	64210	18	LR	Bale	Dinsh	3400	17	3978	22	LS	Arsi	Sude	2570

N.B : Early (<79 DFL), Medium (79 < DFL < 90), Late (DFL>90), resistant (scald < 60)

ER= Early & Resistant, LR= Late & Resistant, MR= Medium & Resistant

3.2. Test Environments

Ethiopia is situated in the horn of Africa as lies between latitudes 3° 15' and 18° North of the equator; and between 33-48° E. It is bordered on the north and northeast by Eritrea, on the east by Djibouti and Somalia, on the south by Kenya, on the northwest by Sudan and on the southwest by South Sudan. It has diversified agro-ecology. This study was carried out at four barley growing areas in Ethiopia where leaf scald disease is a common problem.

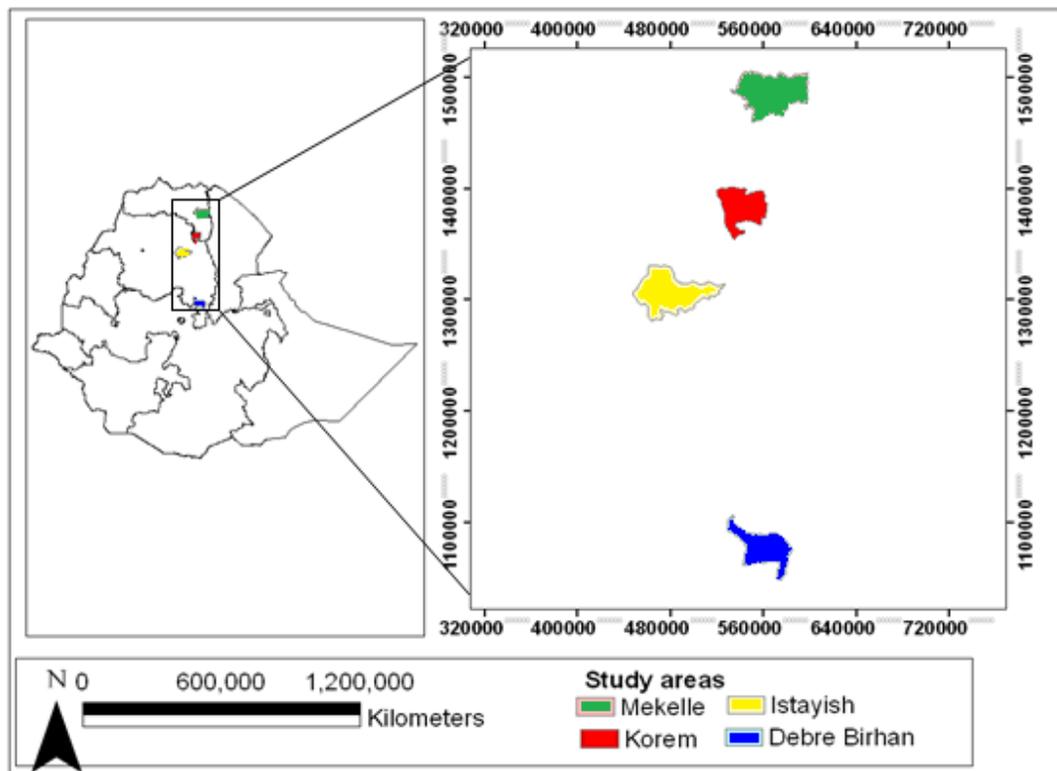


Figure 3: Map of Ethiopia showing specific locations of the study Area

Table 2. Summary of meteorological data for four locations

Site	Altitude (m.a.s.l)	Annual RF (mm)	Min. & Max. Temp (°C)
Mekelle	2,150	300	14.3 - 25.5
Korem	2,530	999	8.8 - 22.8
Debre-Birhan	2,808	1,200	5.5 - 20.6
Istayish	3,294	1,073	2.5 - 18.7

3.3. Experimental design

The experiment was laid out in a 6x6 simple lattice design (partially balanced design) with two replications and two managements (sprayed and non-sprayed). But the spray treatment was made only for the three sites (Korem, Debre-Birhan and Istayish) for Mekelle there was no spray treatment due to small quantity of seed. And the plot size was 1 m wide and 2.5 m length with a row to row spacing of 20 cm.

3.4. Experimental procedures

The experiment was conducted in such a way that barley seeds were planted at four locations in a simple lattice design with two replications on a plot size of 2.5 m² with five rows of 2.5 m long and 1m wide with spacing of 20 cm between rows. The central three rows were considered for all data recordings. The recommended fertilizer rate of 50 kg ha⁻¹ DAP (Diammonium phosphate) and 100 kg ha⁻¹ Urea have been applied during planting in all locations with a seed rate of 90 kg ha⁻¹. In the spray treatment, all genotypes in the two replications were sprayed four times every other two weeks starting from tillering stage with commercially registered anti-fungal chemical known as Tilt 250 EC containing active ingredient propiconazol 250 g/lit at a rate 0.5lit ha⁻¹ to control the development of scald disease. In the non-sprayed treatment, a repeated experiment with the same replication was left free, in other wards all the genotypes have been exposed to natural infestation with no spray. Additionally, outside 2 rows in each plot were planted with susceptible genotype known as “sabine” obtained from Debre Birhan Agricultural Research Center, to enhance disease dissemination. And each genotype was planted by drilling the seeds on a 2.5m² plots of three rows.



Figure 4: Pictures showing chemical (Tilt) spray and infector rows at each plot

3.5. Methods of data collection

Data related to different agronomic traits such as days to heading, days to maturity, plant height, tillering capacity, spike length, seed per spike, thousand seed weight, biological yield, grain yield and harvest index were collected from each plot. Moreover, a disease related data such as scald disease, and net blotch was also collected. Heading date (earliness) was recorded as when the spikes of 50% of the culms had fully come out. Maturity date was recorded when the plants had almost lost their green color from both vegetative and reproductive tissues. Additionally, plant height in cm was recorded, for 5 randomly selected plants from the central 3 rows per plot. And measurement was done from the ground level to the tip of the longest tiller spike excluding the awns after physiological maturity had been reached. Productive tillering capacity was measured when it reached physiological maturity. Spike length (SL) in cm was recorded as height from the bottom to the tip of the spike excluding own. Seed per spike (SPS) was also counted during harvesting from individual sampled plant. Grain yield data was recorded on well dried and of course clean samples from each plot (g/plot). Subsequently grain yield from each plot was converted to tonne per hectare (tha^{-1}). Biological yield was also recorded after harvest for each plot in gram then plot data was converted to tonne per hectare (tha^{-1}). Furthermore, thousand seed weight was measured after adjusting the moisture content to 12.5% on 200 seeds and the final result was multiplied by 5 to get thousand seed weight (gm). Harvest index was also calculated as the ratio of grain yield to biological yield.

3.5.1. Disease Assessment

The level of infection caused by scald diseases (*Rhynchosporium secalis* (Oudem) J.J Davis) was visually scored using double digit scale (00-99) using the modified scale of Saari and Prescott's severity scale to assess wheat foliar diseases (Saari, 1975, Eyal, *et al.*, 1987) where, the first digit (D1) refers to the disease progress in height and the second digit (D2) refers to severity measured as diseased leaf area. So based on the scaling procedure, 00 designated an absence of visible scald lesions, 11 a few isolated lesions on the lowest leaves, and the subsequent degrees in the scale describe increasing levels of scald severity, with 99 indicating a very severe infection all over the plants (over 80% diseased). In this study, from each plot 10 plants were randomly

marked with red string for continuous assessment. As result, diseased leaf area (DLA) was calculated to serve as a basis for estimating area under disease progress curve.

$$DLA = \left[\left(\frac{D1}{9} \right) * \left(\frac{D2}{9} \right) \right] * 100$$



Figure 5: experimental plot during proper identification of the symptom and severity assessment

This was done to quantify the amount of disease as well as the rate of progress over time. Hence genotypes were assessed four times in 15 days interval, corresponding to 4 stages: at tillering stage (Z29), at booting stage (Z45), at anthesis stage (Z60) and milk development stage (Z75) for the percentage leaf area infected during the growing season based on Zadok scale (Zadoks, *et al.*, 1974).

Area under the disease progress curve (AUDPC) was then calculated (Laurence and Madden, 2007) as shown below: AUDPC is usually used to quantify disease intensity over time. It is helpful because it combines the amount of disease over time.

$$AUDPC = \sum_{i=1}^{N_i-1} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i)$$

Where, y_i = disease severity on the i^{th} date, t_i = i^{th} day, and n = number of scoring dates. Then the average disease severity at the midpoint between each two time points was calculated and multiplied that average by the length of time between the two points and summed those products across all time intervals.

Trapezoidal method is commonly used to measure AUDPC as described by (Laurence and Madden, 2007).

Steps:

- The first two infection percentages recorded was added.
- The addition result was divided by two to find the average or mid-value of the two readings.
- The average or mid-value was multiplied by the time interval, which is the number of days from the first reading to the second reading.
- The result was recorded in units of percentage days. The value is an area of a trapezoid.
- Steps were repeated one through four until areas of trapezoid calculation for all readings completed.
- All the area of trapezoids was added to find the AUDPC. Lower AUDPCs represent slower disease progression and greater resistance to the disease. Higher AUDPCs represent faster disease progression and higher susceptibility to the disease.

Table 3: AUDPC based on disease severity measures made at weeks intervals 0, 2, 4, and 6

Sample	Week	Severity	Time interval	Avg. Severity	Time*Severity
to	0	A			
t1	2	B	2	$\frac{a+b}{2}$	$2 * \left(\frac{a+b}{2}\right)$
t2	4	C	2	$\frac{b+c}{2}$	$2 * \left(\frac{b+c}{2}\right)$
t3	6	D	2	$\frac{c+d}{2}$	$2 * \left(\frac{c+d}{2}\right)$

$$AUDPC = \sum_{i=1}^{N_i-1} 2 * \left(\frac{a+b}{2}\right) + 2 * \left(\frac{b+c}{2}\right) + 2 * \left(\frac{c+d}{2}\right)$$

Finally the average scores have been classified based on Saari and Prescott description (Saari and Prescott, 1975) as resistance (≤ 40), moderately resistant (50 to 60) and susceptible (> 70). Furthermore, disease incidence was also recorded just by putting 0.5cm x 0.5cm (0.25cm²) quadrant randomly to each plot. Then total number of plants inside the quadrant was first counted and then identified the diseased plant from the non-diseased ones. Finally, disease incidence was calculated using the formula;

$$SDI\% = \left(\frac{\text{Number of diseased plants in the sample area (0.25m}^2)}{\text{Total number of plants in the sample area (0.25m}^2)} \right) * 100$$

3.6 Data Analysis

3.6.1. Analysis of Variance

Prior to data analysis, quantitative data set have been verified whether it is normally distributed or not (Shapiro and Wilk, 1965). All data obtained was normally distributed. Consequently, each site was individually analyzed using linear mixed model (REML) considering management and genotype as fixed and replication within management and block within replication nested random effects (GenStat 12th Edition).

Table 4: Skeleton ANOVA table for individual site

Sources of variation	df	F-test denominator	Expected Mean Square
Mgt	1	R/M	$\sigma_B^2 + 36\sigma_R^2 + 72\sigma_M^2$
Rep/Mgt	2	B/R/M	$\sigma_B^2 + 36\sigma_R^2$
Blocks/Rep/Mgt	20	LEE	σ_B^2
Genotype	35	from SED(Gen)	$\sigma_L^2 + 4\sigma_G^2$
Mgt x Genotype	35	LEE	$\sigma_L^2 + 2\sigma_{MG}^2$
Residual	50		σ^2
LEE			
Total	143		

Mg^t=management, Rep.= Replication

$$R_{kilr} = \bar{X} + M_k + G_i + MG_{ki} + R_l(M_k) + B_r/R_l(M_k) + e_{kilr}$$

The observed response R_{kilr} of the genotype i in the management k , and block r : Where \bar{X} = grand mean; M = management, G = genotype, MG =management by genotype interaction, $R / (M)$ = replication within management, $B/R / (M)$ = block and replication within management and e_{kilr} = error term.

$$R_{kijlr} = \bar{X} + M_k + G_i + MG_{ki} + L_j + LM_{jK} + R_l / (LM_{jK}) + B_r/R_l / (LM_{jK}) + LG_{ji} + LMG_{jki} + e_{kijlr}$$

The observed response R_{ijkr} of the genotype i in the management k , location j , and block r : Where \bar{X} = grand mean; M = management, G = genotype, MG =management by genotype interaction, L = location, LM = location by management interaction, $R / (LM)$ = replication within location by management interaction, $B/R / (LM)$ = block and replication nested under location by management interaction and e_{kijlr} = error term.

Table 5: Skeleton ANOVA table for across sites

Sources of variation	Df	F-test denominator	Expected Mean Square
Loc	2	R/M	$\sigma_B^2 + 36\sigma_R^2 + 144\sigma_L^2$
Mgt	1	L*M	$\sigma_B^2 + 36\sigma_R^2 + 72\sigma_{LM}^2 + 216\sigma_M^2$
LocxMgt	2	R/(L*M)	$\sigma_B^2 + 36\sigma_R^2 + 72\sigma_{LM}^2$
Rep/(Loc*Mgt)	6	LEE	$\sigma_B^2 + 36\sigma_R^2$
Blocks/Rep/(Loc*Mgt)	60	LEE	
Genotype	35	L*G	$\sigma_L^2 + 4\sigma_{LG}^2 + 12\sigma_G^2$
Loc * Genotype	70	LEE	$\sigma_L^2 + 4\sigma_{LG}^2$
Mgt * Genotype	35	L*M*G	$\sigma_L^2 + 2\sigma_{LMG}^2 + 6\sigma_{MG}^2$
Loc * Mgt * Genotype	70	LEE	$\sigma_L^2 + 2\sigma_{LMG}^2$
Residual	150		σ^2
LEE			σ_L^2
Total	431		

Mg't = management, Rep. = Replication, Loc. = Location

Management, genotypes and management by genotype interaction was fitted as fixed model (Mgt + Genotype + Mgt x Genotype) where as location, location by management interaction, replication within location by management interaction and block within replication, location by genotype interaction and location, management, genotype interaction was fitted as random model (Loc + Loc*Mgt + Loc.Mgt.Rep + Loc.Mgt.Rep.Blocks + Loc*Genotype + Loc * Mgt * Genotype). Lattice effective error (LEE) was also calculated from the standard error of the difference $SED = \sqrt{\frac{2*EMS}{r}}$ for traits having larger block within replication mean square as compared to residual mean square for treatment F-test (Cochran, 1957).

Moreover, best liner unbiased predictor (BLUP) values for each traits in each location was estimated using restricted maximum likelihood (REML) to accommodate field plot heterogeneity which was modeled using spatial covariance structures considering row and column effect (Payne, 2009). The effects of genotypes and spray treatment were modeled as fixed in this case. This BLUP values were actually used in correlation test, and descriptive statistics.

Variance component

Variance component of the different sources of variations for each traits were first calculated. Genotype variance component for example was calculated by subtracting error mean square from

genotype mean square and divided by the product of number of locations, replications and managements which is 12:

$$G_{ms} = \sigma^2_e + 4\sigma^2_{GE} + 12\sigma^2_G$$

Where, G_{ms} = genotypes mean square, σ^2_e = error mean square, σ^2_{GE} = genotype by environment interaction variance component and σ^2_G = genotype variance component.

Heritability (H)

Broad-sense heritability (H) was calculated in order to know proportion of phenotypic variation in genotype means that is due to genotypic differences based on entry mean across environments from the variance components of genotype and genotype by environment interactions using the following formula:

$$H = \frac{\sigma^2_g}{\sigma^2_g + (\sigma^2_{gxe})/r + (\sigma^2_e)/r * e * m} = \frac{\sigma^2_g}{\sigma^2_g + (\sigma^2_{gxe})/3 + (\sigma^2_e)/12}$$

Where H = broad-sense heritability which reflects all the genetic contributions to a population's phenotypic variance including additive, dominant, and epistatic (multi-genic interactions), σ^2_G = the total genetic variance of the genotypes, $(\sigma^2_{gxe})/3$ = genotypes x environment variance which is divided by number of replications and $(\sigma^2_e)/12$ = error variance which is divided by the product of replication, environment and management (spray treatment).

Heritability based on individual plot basis was also calculated using the following formula:

$$H = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_{gxe} + \sigma^2_e}$$

3.6.2. AMMI analysis

Averaged data of grain yield, days to heading, days to maturity and scald disease resistance was subjected to stability analysis over seven environments namely Mekelle, Mekelle-09, Ayba-09, Korem, Debre-Birhan, Debre-Birhan-04 and Istayish. Of which Mekelle, Korem, Debre-Birhan and Istayish were actually the main locations where by data collection carried out in 2012 growing season. However, data obtained from Mekelle University crop research for 2004 and

2009 growing season (Debre-Birhan-04, Ayba-09, and Mekelle-09) were incorporated in order to get strong insight in to the genotype performance pattern across environments. But Debre-Birhan-04 was not included in the yield stability analysis because of absence of grain yield data. Further, Mekelle-09 and Ayba-09 were not included in the disease stability analysis due to again absence of data. So mean of the genotypes for grain yield, heading days, maturity days and disease resistance data of the environments were subjected to AMMI analysis (Gauch, 1992), which is a combination of analysis of variance and multiplication effect analysis, to investigate GE interactions using IRRISTAT software (IRRISTAT, 2007). AMMI analysis subsequently produced ANOVA table and Biplot graph.

The model AMMI equation is:

$$Y_{ij} = \mu + g_i + e_j + \sum_{n=1}^N \lambda_n \gamma_{in} \delta_{jn} + \rho_{ij}$$

where Y_{ij} is the yield of the i^{th} genotype in the j^{th} environment; μ is the grand mean; g_i and e_j are the genotype and environment deviations from the grand mean, respectively; λ_n is the eigenvalue of the IPC analysis axis n ; γ_{in} and δ_{jn} are the genotype and environment eigenvectors for axis n ; n is the number of principal components retained in the model and ρ_{ij} is the error term.

Analysis of variance is usually used to partition variance into three components: genotype deviations from the grand mean, environment deviations from the grand mean, and GE deviations from the grand mean. In AMMI analysis, in addition to genotypic effect multiplication effect analysis is used to partition GE deviations into different interaction principal component axes (IPCA), which can be tested for statistical significance through ANOVA.

The AMMI stability value (ASV) was calculated based on the description of (Purchase, 2000) as follows:

$$ASV = \sqrt{\left[\frac{IPCA1_{\text{sum of square}}}{IPCA2_{\text{sum of square}}} (IPCA1_{\text{score}}) \right]^2 + (IPCA2_{\text{score}})^2}$$

Where, $\frac{SS_{IPCA1}}{SS_{IPCA2}}$ is the weight given to the IPCA1-value by dividing the IPCA1 sum of squares by the IPCA2 sum of squares. The larger the IPCA score, either negative or positive, the more specifically adapted a genotype is to certain environments. Smaller ASV scores indicate a more stable genotype across environments.

3.6.3. Regression and correlation analysis

3.6.3.1. Regression analysis

Linear regression investigates and models the linear relationship between a response (Y) and predictor(s) (X). Both the response and predictors are continuous variables. Particularly, linear regression analysis is often used to:

- . Determine how the response variable changes as particular predictor variable changes
- . Predict the value of the response variable for any value of the predictor variable, or combination of values of the predictor variables

In this study, regression analysis was run for individual sites using minitab software (MinitabInc, 2010). The linear associations between the responses, days to heading, days to maturity and grain yield, and the predictors, scald disease were assessed by linear regression.

$Y_i = \alpha_0 + \beta_1 X_i + (\beta_2 X_j) * 1 = Y_i = \alpha_0 + \beta_1 X_i + \beta_2 X_j$ (Sprayed), there are two independent variables in this case X_i and X_j .

$Y_i = \alpha_0 + \beta_1 X_i + (\beta_2 X_i) * 0 = Y_i = \alpha_0 + \beta_1 X_i$ (non-sprayed), simple linear regression, there is one independent variable: X_i and two parameters α_0 and β_1 for modeling n data points

Where, Y is the dependent variable, α_0 , is the intercept, β , is the slope, and X is the independent variable. In both cases, e_i is an error term and the subscript i index a particular observation for: X_i .

3.6.3.2. Correlation analysis

Correlation analysis was similarly carried out using the averaged BLUP values, calculated by incorporating row and column effects to account for spatial trends (Payne, *et al.*, 2011), for six environments using minitab software (MinitabInc, 2010). The intention was to assess if there are any associations among the scored traits and to measure the extent to which two continuous variables are linearly related.

3.6.4. Principal component analysis (PCA)

Principal component analysis (PCA) was run using past software (Hammer, 2001) with the objective of reducing the dimensionality (number of variables) of the data set with out changing original variability in the data in order to graphycally visualize either postive or negative associations among traits in a simple way (Figure 35).

Note: AMMI analyses for grain yield included 6 environments (Debre-Birhan-04) was absent due to lack of yield score. For heading day analysis, 7 environments were considered but for disease analysis only 5 environments were incorporated due to absence of disease score for Mekelle-09 and Ayba-09