CHAPTER 4

Materials and methods

The principal objective of tobacco plant breeding programmes is to improve yield per hectare and quality for high economic gains (Stoskopf et. al., 1993; Wallace and Yan, 1998; Simmonds, 1987). Breeding for any desirable characteristic in tobacco can be either a direct or an indirect means of achieving the above objective.

4.1 Background work

Based on the above objective, various crosses of carefully selected parental genotypes have been made by the Tobacco and Cotton Research Institute (TCRI) of the Agricultural Research Council (ARC). Advancement of generations and selections for desirable characteristics have been conducted simultaneously in a series of trials at the ARC-TCRI. The promising breeding lines namely OD1, OD2, OD490, ODT92, ODT19, ODT8, ODT82, OD486B, ODT100, ODT1 and OD313 have been advanced to elite breeding lines (Table 4.1).

4.2 Current work

In the current work, the elite breeding lines were evaluated for their field and market performance. The days that the plants take from transplanting to physiological maturity, plant photosynthetic competence, plant height at topping, number of leaves per plant, leaf areas and yield were investigated as parameters of crop field performance. Cured leaf colours, nicotine and reducing sugar concentrations of the leaf, grade indices of the cured leaf and the monetary returns per hectare were investigated as parameters of crop market performance.

Table 4.1: The elite breeding lines and their pedigrees

Elite breeding line	Pedigree
OD1	(C411 x TL33) x (SpG28 x TL33)
OD2	17/17/8/30 x OD212/8
OD490	SpG80 x 10/43
ODT92	K326 x TL9hon
ODT19	Island Gold x M1
ODT8	K326 x 33/94 sub
ODT82	K326 x OD272
OD486B	17/17/8/30 x OD212/8
ODT100	K326 x OD212
ODT1	Island Gold x OD272
OD313	Virginia 115 x {(C347 x TL33) x (SpG28 x TL33)}
TL33 (Control)	SpG28 x A23

OD2 and OD486B are different selections from the same parentage.

4.3 Tobacco nursery management

Seedling production involved sowing tobacco seeds in a complex germination medium made of vermiculite, peatmoss and perlite in compartmentalised seed-germination trays in a glasshouse. The tray compartments were filled with the germination medium and watered lightly. 0.2g of tobacco seed of a particular genotype was then broadcast onto the compartmentalized seedling tray by hand.

An automated overhead spray-irrigation system was used to water the nursery. This watering system is used at the ARC-TCRI because it demands a lower labour requirement than the use of pipes and watering cans. Soon after sowing, the watering was light and frequent. The watering became heavier but less frequent as the seedlings grew to keep seedling growth rate steady and constant.

After germination, when the seedlings were about five centimetres tall, they were thinned and spaced to one seedling per compartment to reduce competition so that a strong, healthy and uniform stand of 128 seedlings per tray could be achieved.

Nursery fertilizers, fungicides, insecticides and a surfactant were administered by drenching according to Schedule 4.1. The fertilizers included Multifeed™, calcium nitrate (C_aNO_3) and limestone ammonium nitrate (LAN). Apron™C and Virikop™ are the fungicides that were used to combat powdery mildew, wildfire and damping-off. Baytan™ was used to control systemic fungal disease-causal agents. Orthene™ and Endosulfan™ are the insecticides that were used to keep insect damage under control. Bladbuff™, a surfactant, was used to enable the chemicals to stick to the leaf surfaces for a lengthy effective action.

Schedule 4.1: Fertilizer application, and disease and insect pest control programme used in the seed tray nursery.

Chemical and quantity	Day										
	1	7	14	21	28	35	42	49	56	63	70
C_aNO_3 400g	+	+	+	+	+	+	+	+	+	+	+
Multifeed™ 50g		+	+	+	+	+	+	+	+	+	+
LAN 208g			+	+		+	+	+		+	+
KNO_3 224g					+			+			
Apron™C 30g	+			+			+			+	
Baytan™ 8ml		+			+			+			+
Virikop™ 100g						+			+		
Endosulfan™ 40g				+				+			
Orthene™ 80g		+				+				+	
Bladbuff™ 20ml	+	+			+	+	+	+	+	+	+
Water 40ml	+	+	+	+	+	+	+	+	+	+	+

The drenching programme started one week after the thinning and spacing of the seedlings in the seed trays. The chemical mixture was applied at the rate of 15 litres per 50 seedling trays that covered 11.39 square metres of nursery area. Each tray contained 128 seedlings.

Weeding was done by pulling the weeds out as soon as the weeds appeared. A pair of forceps was used to pull out the weeds before the weeds developed large root volumes. Weeds with large roots damage the tobacco seedlings in the process of weeding.

The top leaves of the seedlings were clipped off regularly to maintain uniformity in seedling height within a genotype in a seed tray and to rejuvenate the fast-growing genotypes. A lawn mower fitted with height adjusters and suspended on a bench was used to clip the seedlings. The seedling tray was pushed across the bench but under the running machine to clip off the top leaves of the seedlings. The height of the machine was adjusted depending on the general height of the seedlings in the tray to avoid damaging the apical meristems. Rejuvenating the fast-growing genotypes and maintaining uniformity in seedling heights were important to avoid unnecessary variation in the experimental seedlings.

Three weeks before transplanting, the seedlings were taken from the glass house into the open and deprived of water to harden them off. Hardening off enables the seedlings to survive the shocks of transplanting and the harsh conditions of the field. A well-hardened seedling would not snap easily when bent. Hardened seedlings withstand the handling pressure in the processes of transporting them from the nursery to the field and transplanting.

4.4 Land preparation

The trial was conducted on 0.1 hectare of land at the ARC-TCRI. The ARC-TCRI is located at Kroondal near Rustenburg in the North West Province of South

Africa. Rustenburg is located at an altitude of 1157 metres above sea level, latitude of 25° 43' South and a longitude of 27° 18' East.

The land had been grown with *Setaria sphacelata*, a rotational grass locally known as manna grass, during the previous season. Deep ploughing and discing had been done almost six months before transplanting.

Two sets of soil samples were taken from the land in a random manner at three different soil depths: 0-20cm, 20-40cm and 40-60cm before ridging was done. One of the two soil samples was taken as a back-up sample. The soil depth of up to 60cm was chosen because tobacco is a dicotyledonous plant with a tap root system. Therefore, the growth of a tobacco plant is influenced by the soil condition mostly within this soil depth. The soil samples were sent to the soil chemistry laboratory for soil chemical analysis. The results of soil chemistry analysis are presented in table 4.2.

Table 4.2: Results of soil chemistry analysis (p.p.m. - parts per million).

Soil depth	0-20 cm	20-40 cm	40-60 cm	Average
pH	5.91	6.15	6.26	6.11
% sand	38.00	28.00	27.00	31.00
% silt	8.00	6.00	9.00	7.67
% clay	54.00	66.00	64.00	61.33
N p.p.m.	4.00	2.00	2.00	2.67
P p.p.m.	5.00	1.00	0.00	2.00
K p.p.m.	163.00	88.00	83.00	111.33
Ca p.p.m.	773.00	680.00	580.00	677.67
Mg p.p.m.	1080.00	1400.00	1480.00	1320.00
Na p.p.m.	8.00	13.00	15.00	12.00
Cl p.p.m.	1.00	1.00	1.00	1.00
Zn p.p.m.	1.44	0.60	0.56	0.87

According to Akehurst, 1968, about 70% of the tobacco crop in South Africa is grown on soils with 50-60% clay, 20-35% sand and 5-30% silt.

Flue-cured tobacco crop favours a pH range of 5.5-6.5 (Akehurst, 1968). According to the soil sample analytical results, the pH value was within the favourable range for the growth of flue-cured tobacco crop. Therefore, there was no need for soil pH adjustments before conducting the trial.

Table 4.2 shows that the soil at this trial site is a clay soil. Soil nematode counting is not a routine for the heavy clay soils of ARC-TCRI because nematodes are most prevalent on light, sandy soils (Akehurst, 1968; and Van Biljon, personal communication). In tobacco, the threshold level of nematode infestation per 100 cubic centimetres of soil, when that nematode species occurs alone, is very low. The threshold level is even lower when two or more nematode genera are present. Crops, like tobacco, that are highly susceptible to nematode infestation allow a rapid build-up of nematode populations from the lowest population densities at transplanting (Keetch and Heyns, 1982; and Clayton, 1958). Based on this notion, a precautionary measure was taken by applying 12 kg of Temik™ G per hectare by banding at the time of ridging to control nematodes that might have possibly been in the soil.

Nitrogen, phosphorus and potassium are the most important major nutritional elements for flue-cured tobacco. The residual amounts (Table 4.2) detected in the soil at the trial site were quite negligible considering the total flue-cured tobacco requirements for proper growth and productivity. Table 4.3 shows the average mineral removals from the soil by a flue-cured tobacco crop, which gave an average yield level of 3769.22 kg/ha (Van Dierendonck 1959). This yield level would be acceptable at the ARC-TCRI and other similar ecological zones even now, considering the average yield level of the currently accepted cultivar, TL33 (2986.22 kg/ha) (Table 5.6).

Table 4.3: Average mineral removals (kg/ha) from the soil by a flue-cured tobacco crop (Dierendonck, 1959)

Mineral	N	P	K	Ca	Mg	Crop yield (kg/ha)
Quantity (kg)	74	22	133	106	27	3769.22

Fertilizer application regime in this trial was aimed at meeting the flue-cured tobacco fertilizer requirements as much as possible. Therefore, Schedule 4.2 was formulated and followed accordingly.

Table 4.4: Contribution of applied fertilizers to N, P and K levels (kg/ha) in the soil according to Schedule 4.2.

Fertilizer		2:3:4: (33)	LAN	KNO3	1:0:1 (38)	LAN	Total
Contribution	N	48.00	42.84	12.32	17.48	21.84	142.48
	P	96.00	00.00	00.00	00.00	00.00	96.00
	K	120.00	00.00	12.32	17.48	00.00	149.80

The values of the applied N, P and K were higher than those recorded by Dierendonck. A fertilizer regime that would give relatively higher N, P and K values (Table 4.4) was chosen to allow for nutrient losses. Despite the low rainfall patterns at the ARC-TCRI exemplified by the rainfall during this season (Figure 4.2), the rains come in heavy storms that tend to be erosive, and likely to cause loss of nutrients through run-off and leaching.

Calcium, sulphur and magnesium are rarely deficient in most soils and their importance is masked by the quantities included in the nitrogen, phosphorus and potassium fertilizer compounds. Trace elements are also rarely deficient (Akehurst, 1968). Therefore, calcium, sulphur, magnesium and the trace elements were not considered in the formulation of the fertilizer application regime.

Ridges were made 120cm apart simultaneously with basal dressing fertilizer application (Schedule 4.2) and Temik™ application at 12 kg/ha for a precautionary measure against nematodes. The trial was laid out in a randomised design, replicated three times (Figure 4.1). The randomisation was done by using AGROBASE version of randomisation. Each gross plot consisted of five ridges. Each ridge was five metres long. Planting holes were marked 50cm apart along the ridges.

At the northern and eastern ends of the trial, there were other tobacco trials. On the southern end, the trial bordered on a natural bush. On the western end, there was a rotational crop, sunhemp.

3	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**	**33**	**36**
102	106	109	112	208	210	212	202	304	305	307	310
2	**5**	**8**	**11**	**14**	**17**	**20**	**23**	**26**	**29**	**32**	**35**
111	103	107	110	205	201	206	204	306	303	312	301
1	**4**	**7**	**10**	**13**	**16**	**19**	**22**	**25**	**28**	**31**	**34**
104	108	105	101	203	209	207	211	309	311	302	308

→ North

Figure 4.1: Experimental field plan – Randomised design

The numbers **1-36** represent plot numbers. The numbers 101-112, 201-212, 301-312 represent replications and treatments. The first digits represent the replication numbers, while the second and the third digits together represent the treatment numbers (Table 4.5).

Table 4.5: The treatment (T) numbers and the corresponding genotypes (G).

T	01	02	03	04	05	06	07	08	09	10	11	12
G	OD 1	OD 2	OD 486B	TL 33	OD 490	ODT 92	ODT 1	ODT 100	OD 313	ODT 19	ODT 8	ODT 82

4.5 Transplanting

Tobacco needs to be transplanted in the field when the soil is at field capacity. The soil moisture at transplanting (November 26, 1998) was equivalent to approximately 103.4 mm/30 days=3.4mm of rainfall per day (Figure 4.2). This soil moisture would not meet the initial crop water-requirements.

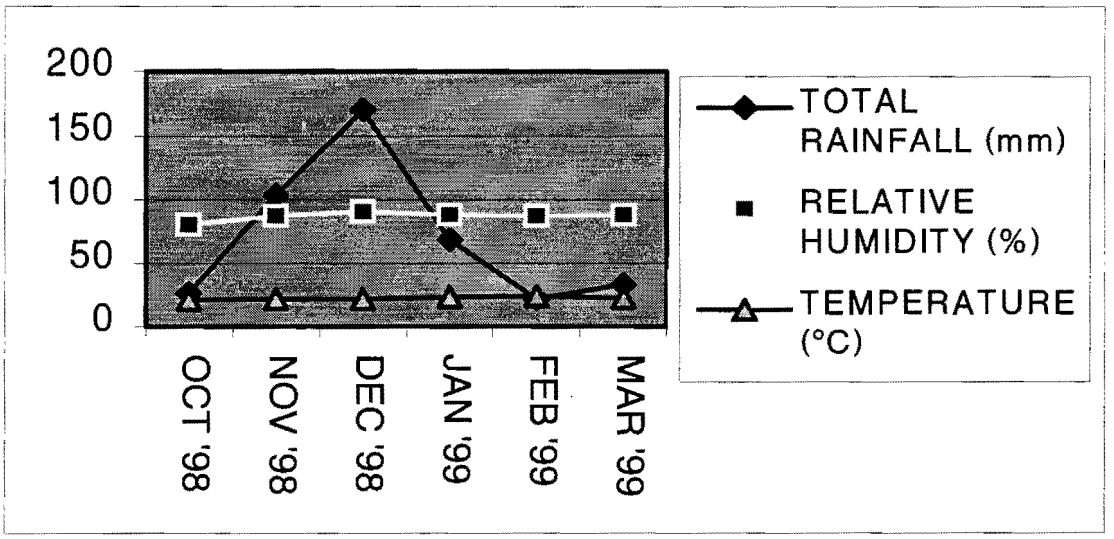


Figure 4.2: Weather pattern at the ARC-TCRI during 1998/1999 season.

The y-axis has a common scale of 0-200.

Therefore, arrangements were made for the trial to be water-transplanted. One to two litres of water was poured from a water pipe into each planting hole. Immediately, a planter holding the seedling tray in the left hand used the right hand to pull out from the tray a seedling with an intact mass of nursery growth medium around the roots. The seedling was centrally stuck into the wet soil at the bottom of the planting hole, making sure that the meristem was not submerged in water. As soon as the water was absorbed into the soil at the base of the planting hole, the planting hole was filled with dry soil with the seedling shoot at the centre free of water and mud.

In this manner, ten seedlings were transplanted per ridge. Therefore, with the five ridges per plot, the plot consisted of 50 plants.

Black shank is a disease that is prevalent in the soils of the ARC-TCRI. Therefore, 360g of Ridomil™ WP mixed with 15 litres of water was applied at the rate of 50 millilitres of the mixture per plant after the transplanting operation to control the disease. This application rate required 20.83 kg of Ridomil™ WP per hectare.

4.6 Field management

25-38mm of rainfall in a seven to ten-day period, 30-32°C of day temperatures and 18-21°C of night temperatures are necessary conditions for maximum growth of tobacco plants (Hawks, 1978; and Collins and Hawks, 1993). The rainfall regime at the ARC-TCRI (Figure 4.2) was inadequate to meet the crop-water requirement. Therefore, sprinkler irrigation was used, whenever necessary, to supplement the inadequate rainfall. The temperatures were within the required ranges for flue-cured tobacco production (Akehurst, 1968; and Hawks, 1970).
According to the nutritional requirement of flue-cured tobacco (Table 4.3), and the residual nutrient status of the trial site (Table 4.2), Schedule 4.2 for fertilizer application was followed. Split fertilizer application procedure was adopted to minimise excessive nutrient leaching losses that would possibly occur if all the required nutrients were applied in one dose. Akehurst (1968) underlined the necessity of adopting the split application procedure to ensure correct soil nutritional status that influences tobacco cured leaf quality.

Schedule 4.2: Fertilizer application regime

Fertilizer	Application rate (kg/Ha)	Time of application	Type of Application	Mode of application
2:3:4 (33)	800	At ridging	Basal dressing	Banding
LAN	153	3 weeks AT*	First top dressing	Dollop
KNO3	88	6 weeks AT*	Second top dressing	Dollop
1:0:1 (38)	92	9 weeks AT*	Third top dressing	Dollop
LAN	78	12 weeks AT*	Fourth top dressing	Dollop

AT* = After transplanting

Weeding was done regularly by means of a hoe. When the crop canopy became so closed that using a hoe would lead to leaf damage or leaf drop, any weeds that persisted were pulled out by hand.

When 50% of the plants in any one plot reached the extended bud stage of flowering, the plants were topped. The extended bud along with the flag leaf was clipped off at an angle to prevent stagnation of suckercide on the cut surface. The suckercide, Fair 85™, diluted at 150ml/5 litres of water, was applied at the rate of 8ml per plant (3.88 litres/ha) on the cut surface. The topping angle allowed the suckercide to flow down the stem, 'burning' the axillary buds, until the bottom-most axillary bud. Any surviving suckers were removed by hand.

4.7 Field data collection

Observations of plant and leaf characteristics were recorded from ten randomly selected plants in each net plot. The plant and leaf characteristics included days from transplanting to physiological maturity, chlorophyll ***a*** fluorescence and plant height at topping. Numbers of leaves per plant and leaf lengths and widths were recorded. Leaf areas (LA) were derived from the formula: LA = LL x LWm x factor

(Suggs et al, 1960), where LL = leaf length, LWm = maximum leaf width, and the factor was 0.634. Whole-plant leaf area (WPLA) was calculated using the formula: WPLA = LA x NLP where LA = mean (single) leaf area, and NLP = number of leaves per plant.

4.8 The visual evaluation of the elite breeding lines by the representatives of the tobacco industry.

Representatives of the tobacco growers' cooperatives, extension officers, leaf dealers and manufacturers were invited to evaluate the different elite breeding lines at the ARC-TCRI. Unfortunately, this particular trial was not evaluated because of time constraint. However, in a similar exercise conducted in 1997/1998 growing season, a similar trial with some of its entries also found in this trial, was evaluated. The representatives recorded the scores of the elite breeding lines on evaluation cards (Appendix 3) using the scale of 1-5. One represents a line, which is totally unacceptable. Five represents a perfect line.

4.9 Harvesting of the tobacco leaf

Signs of maturity start to appear in the bottom-most leaves. The signs include yellowing of the leaf; the drooping of the leaf to a right or an obtuse angle with the stem; and twisting and curling of the leaf. A dull, mottled and wrinkled surface of the leaf (Photographs in Appendix 8) and the brittleness of the lamina were yet other easily noticeable signs of the maturity of the leaf.

Harvesting of the leaf was started seven days after the last topping operation. One reaping was done per week. Three to four bottom-most leaves were plucked at every reaping. The harvested leaves were clipped in bulk racks. The racks with the tobacco leaf were suspended on rails mounted on a tractor-trailer ready for transportation to the curing ovens. The harvested tobacco leaf was always covered to protect it against the scorching effect of the hot sun. Harvesting of the

leaf was completed in six reaping operations.

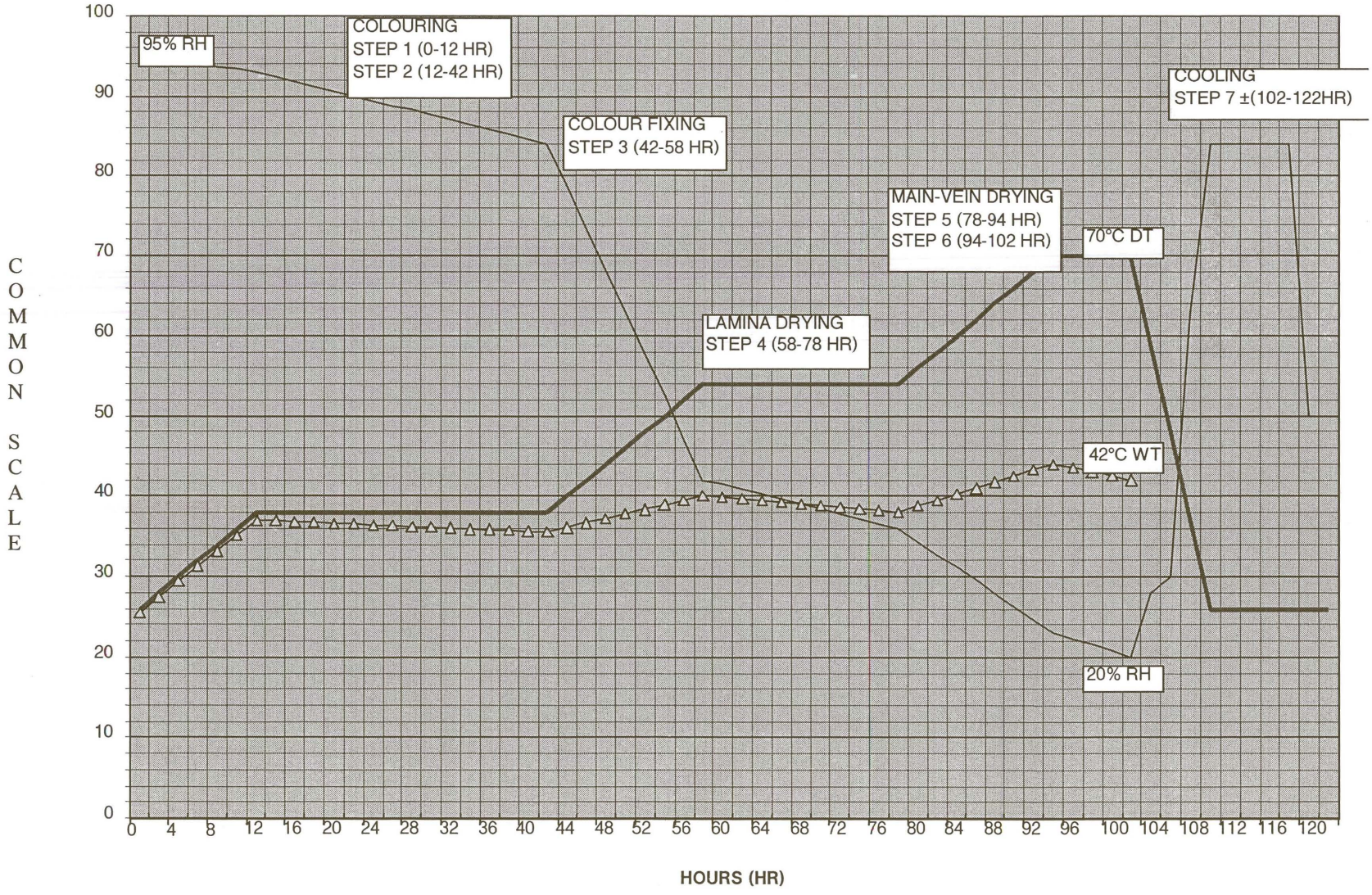
4.10 Post-harvest management of the tobacco leaf

The racks with the tobacco leaf were packed in a bulk oven until the oven was full. The bulk oven operates on a hot-water system. The curing of the leaf was accomplished by means of hot air, pre-heated by a water heat exchanger, using coal as fuel. Schedule 4.3 was used as a guide to the tobacco curing procedure.

Schedule 4.3: A guide to the curing procedure of flue-cured tobacco

DT Dry bulb thermometer temperature reading (°C) WT Wet bulb thermometer temperature reading (°C)

RH Relative humidity (%)



The cured leaf is normally brittle. However, it is highly hygroscopic. Therefore, the leaf was conditioned by mist sprays in readiness for grading.

The grading was done according to leaf colours, quality degree, curing pattern, body, grain, maturity, damage and the position of the leaf on the stalk in accordance with the description of the grades (Anonymous, 1968).

The previous year's tobacco grade indices (cents/kg) were used to determine the monetary returns per hectare.

Data on leaf yield, leaf quality expressed as grade indices, and monetary returns were determined from whole net plots and expressed in units per hectare wherever necessary.

Cured leaf samples representative of all leaf positions on the stalk were taken and sent to the biochemistry laboratory for determination of nicotine and reducing sugar concentrations in the leaf.

4.11 Statistical analyses of data

A computer statistical program written by Vorster (personal communication) was used to perform the analyses of variance for all the sets of data. Vorster's program transforms the raw data into tables of means and then uses the tables of means to perform the analyses. The completed analyses show the performance of the treatments and the corresponding statistics, ready for interpretation (Appendices 1, 2, 4, 5, 6, and 7).

The correlation analyses of the parameters of field performance were performed by means of a calculator. The parameters included the days that the plants took to reach physiological maturity, plant height at topping, number of leaves per plant, whole-plant leaf area and yield. The aim of carrying out the correlation

analyses was to isolate the parameters that constitute the most important yield components of the elite breeding lines being tested.

Statistical comparisons of the field and market performance of the elite breeding lines and the control, TL33, were made at the 0.05 level of probability, unless otherwise stated.

The results from a single trial at Rustenburg could not be accurate enough to serve as the basis for meaningful conclusions because of the inherent inaccuracy of a single trial at a single locality. Therefore, further investigation took into account three other similar trials at Groblersdal in Mpumalanga Province, Potgietersrus in the Northern Province and Vaalwater also in the Northern Province.

The plant breeding department of the ARC-TCRI evaluated the same elite breeding lines at the other three aforementioned localities during the same season, 1998/1999. The same experimental design, number of replications, plot sizes and the control cultivar as those used in the trial at Rustenburg were used at the other three localities.

The trials at the other three sites were carried out according to the protocol (unpublished) recorded in 'LNR Tabak Proefboek: Afdeling Teling' (Vorster, 1998) which was, basically, the same as that followed in conducting the trial at Rustenburg. Therefore, it is not necessary to discuss the details of the procedures of the other three similar trials.

With the kind permission of the plant-breeding department of the ARC-TCRI, a combined analysis, using the AGROBASE, was run on yield, quality and income data from the other three localities and those from the trial at Rustenburg.
ARC-TCRI has up to eleven trial sites for the evaluation of the elite breeding lines the choice of which was dictated by the demand from certain tobacco growers'

co-operatives and the differences in climate among the sites. The data from all these sites could not be used in the combined analysis as some trials had entries, which were not included in the trial at Rustenburg. Furthermore, the trials at some sites suffered hail damage so that it would be an unfair comparison towards such sites if they were to be included in the combined analysis.

From the aforementioned factors, it may be understood that the use of data from Groblersdal, Potgietersrus and Vaalwater was not by design, but rather by the mere fact of the unavailability of data from identical trials in other localities.

The combined analysis showed that ODT82, ODT92, OD2 and OD1 performed significantly better than TL33 across the localities and that they could do equally well at all the localities. This generalised observation aroused the curiosity to examine the genotype-locality interaction variance. The Additive Main effects and Multiplicative Interaction (AMMI) analysis was carried out mainly to investigate the genotype-locality interaction variance.