

Varietal Identification through Grow Out Test, Chemical Test and Bio Chemical Test

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Genetic purity

Meaning :

- True to type or genuine
- A count is made of the number of seeds, seedlings or plants that are true to this type
- It decides the maximum potential that can be accessed from the variety and also proves the effectiveness of modern agronomic practices
- Plants in a population of a variety are true to the type for which it has been claimed

Varietal Purity Testing

- To ensure the varietal performance over the generations through maintaining varietal purity during seed multiplication and subsequent handling
- To ensure that the farmers gets the seeds of a variety for which he pays
- Presence of transgene in case of GM crop seeds

Minimum standards for genetic purity for different class of seeds.

SL. No.	CLASS OF SEEDS	PURITY %
1	Breeder Seeds	100%
2	Foundation seeds	99%
3	Certified seeds	98%
4	Hybrids	95%

Factors affecting genetic purity.

1. Natural crossing
2. Mechanical admixtures
3. Random drift
4. Mutation
5. Selective influence of pest and diseases.

(Basra, 2002)

Different methods of genetic purity determination

- ▶ Morphological
- ▶ Chemical
- ▶ Biochemical
- ▶ Molecular

Different Morphological Methods

- ▶ Seed morphology
- ▶ Examination of seedlings
- ▶ Examination in green houses
- ▶ Grow out test

Seed morphology testing

- ▶ Characters like **size and shape of grain, base of lemma, vertical crease hairs, rachilla hairs, deviation of lateral dorsal nerves wrinkling of lemma and palea** etc.
- ▶ The colour characteristics examined under full day light or light of limited spectrum e.g. **ultraviolet light**.
- ▶ Scanning electron microscope for studying differences in seed coat surface and its **inner structure** have also been used in some species.



White
IC201087



Creamish white
IC198323



Mottled
IC198326



Grey
IC5969



Creamish brown
IC202868



Light brown
IC198361



Brown
C-152



Dark brown
IC253270

Seed shape



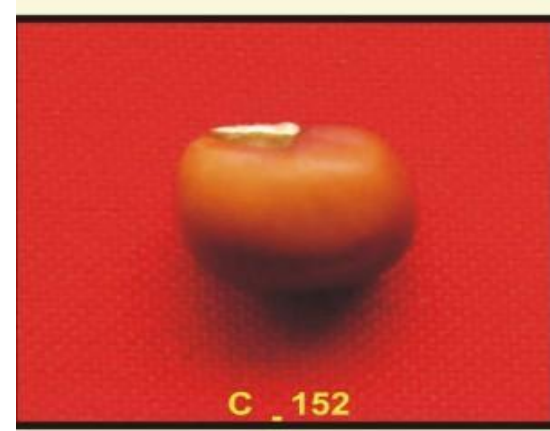
IC253275

Kidney



IC219599

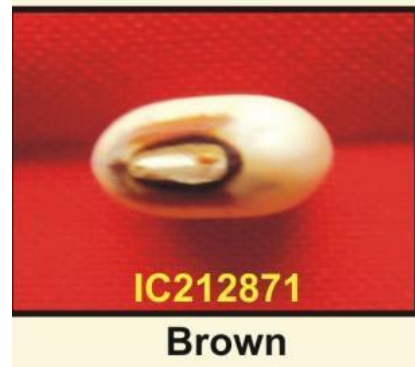
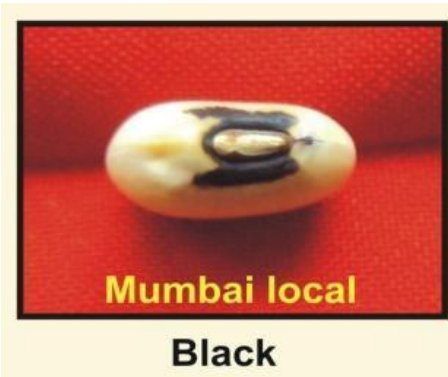
Ovoid



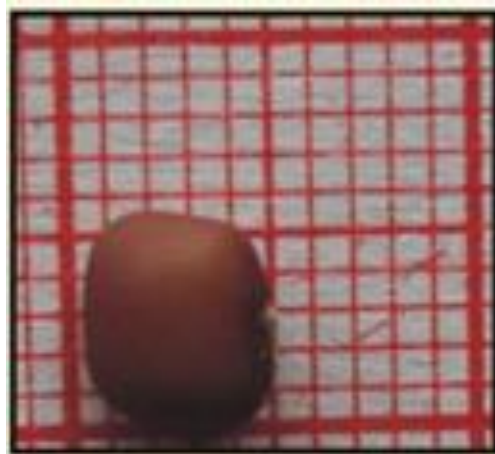
C_152

Rhomboid

Hilum colour



Seed size



Small
IC4506



Medium
IC202867



Bold
IC212872

Traditional approach to purity testing

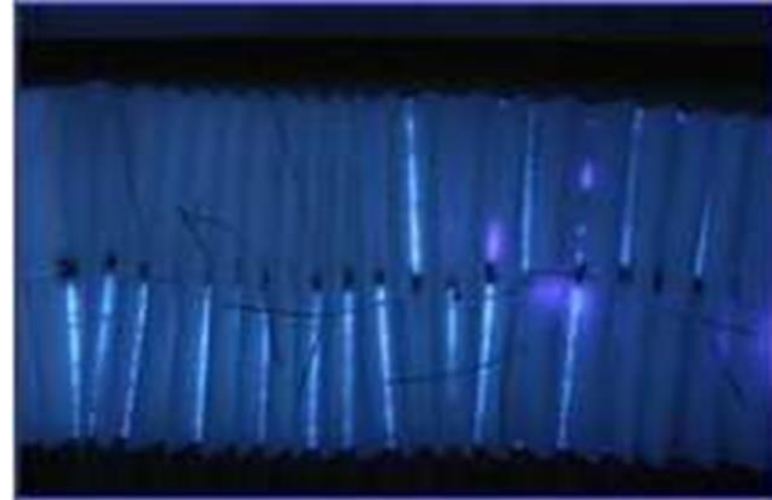
Morphological traits



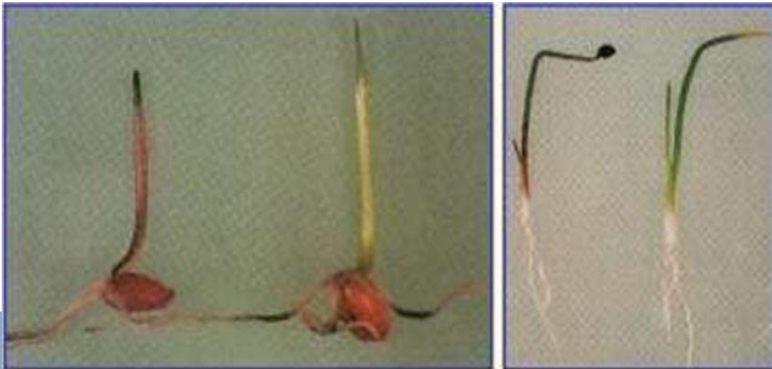
ON SEED



ON SEEDLING



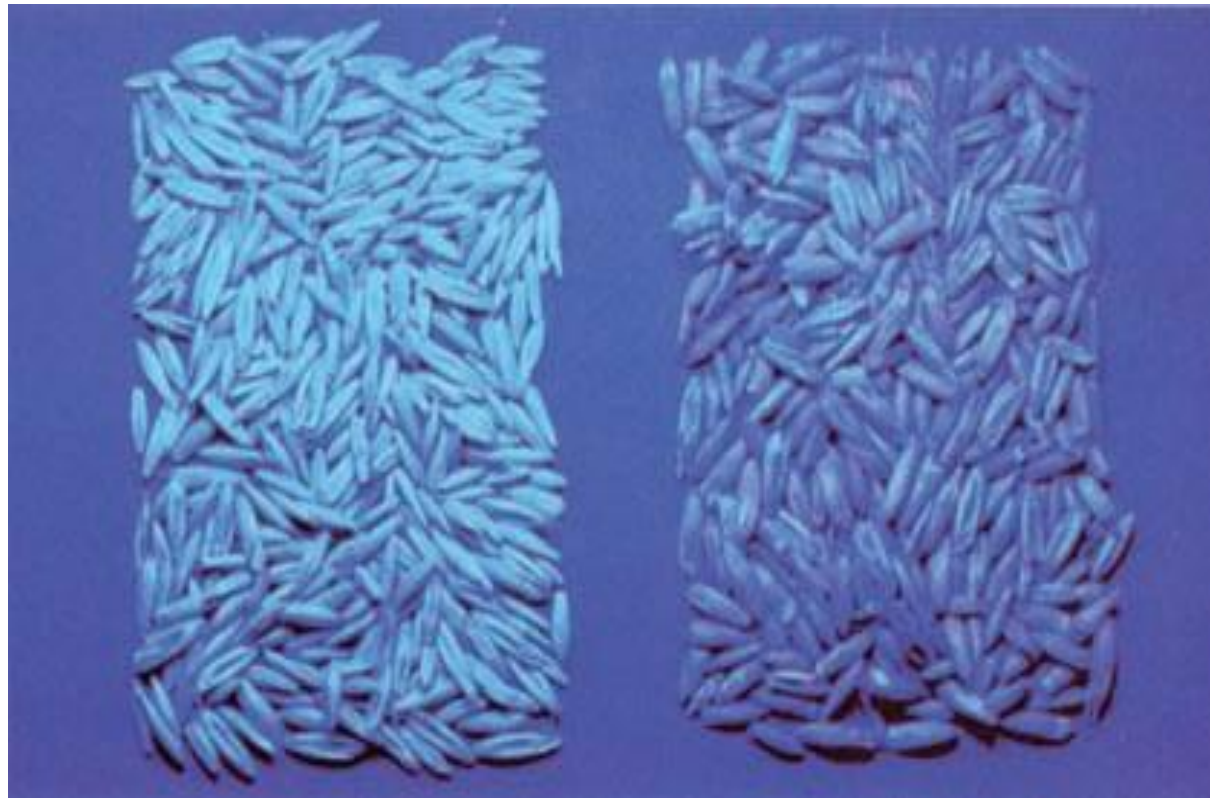
IN LAB OR GREEN HOUSE



IN FIELD



Flourescence (oat)



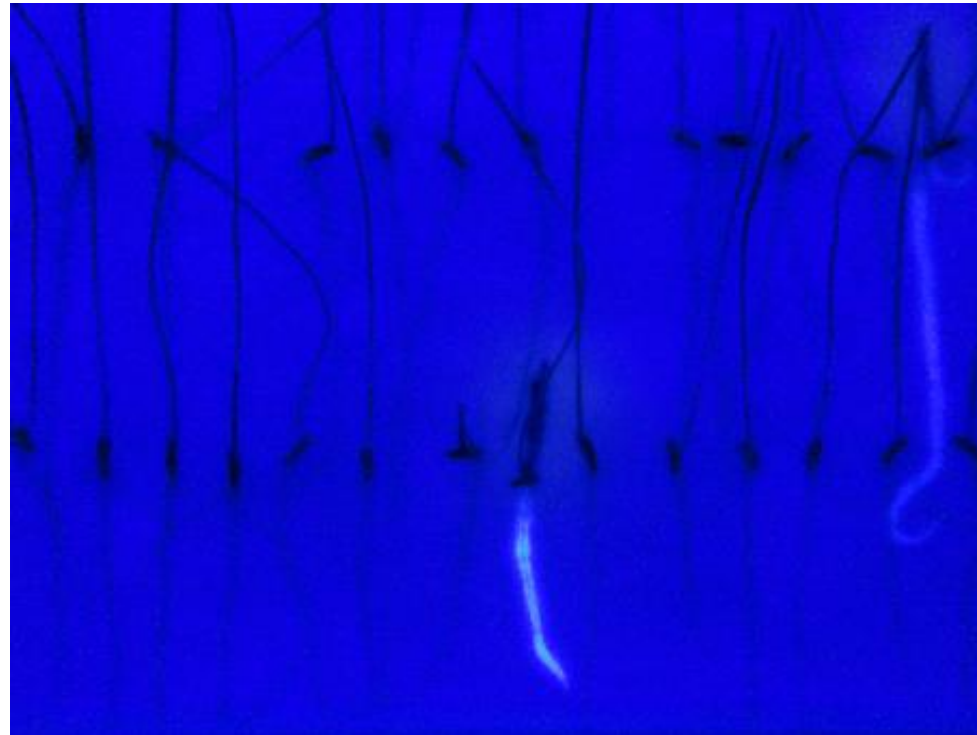
Fluorescence Test (*Lolium*, *Festuca*)

Lolium spp *L. multiflorum*

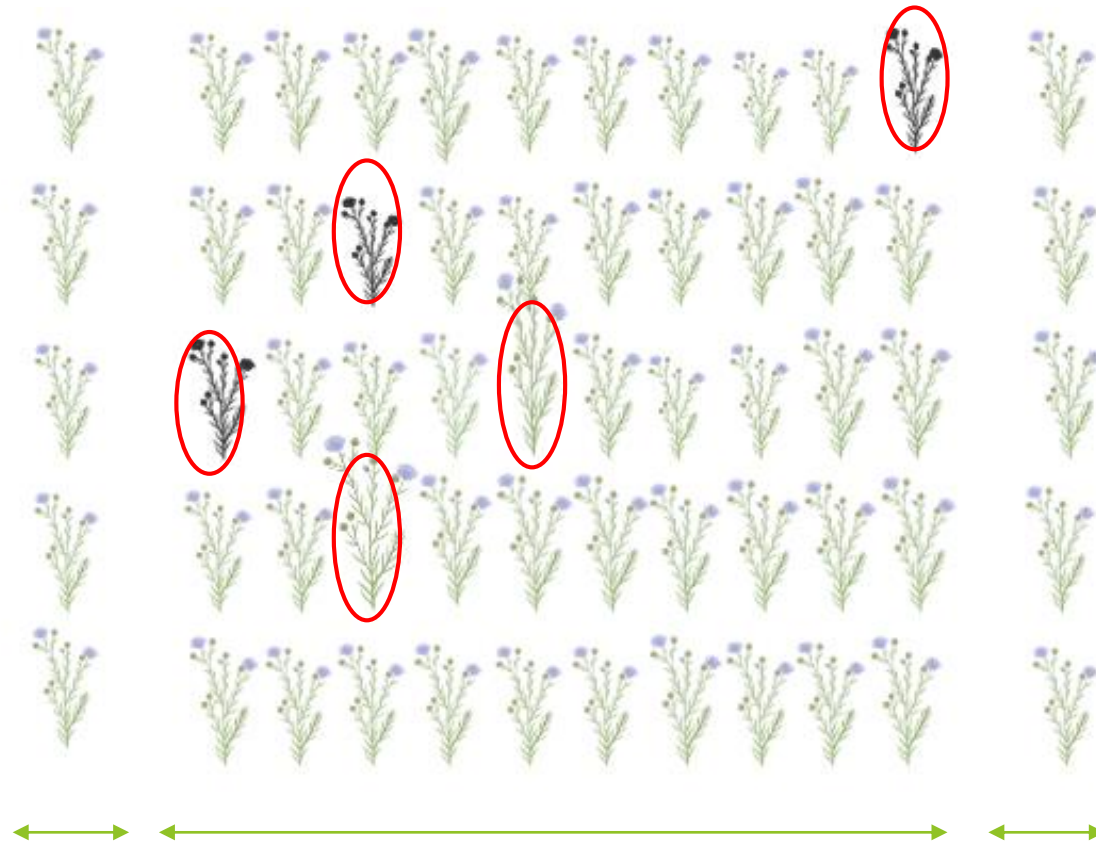
L. perenne

Festuca spp *F. rubra*

F. ovina



Grow out test



Characters

- Highly heritability
 - Stable expression over a range of environments.
 - Easily differentiated by visual observation.
-
- Sufficient spacing between rows and plants.
 - Various samples of the same cultivar sown in succession and standard samples are sown at suitable interval
 - **Deviation** from control sample counted
 - **Mutual comparison between the samples** to be tested and the standard.
 - **Observations at full growing period.**

Sample size(lab and field)

1,000 g -	for maize, cotton, groundnut, soyabean and species of other genera with seeds of similar size;
500 g -	For sorghum, wheat, paddy and species of other genera with seeds of similar size;
250 g -	Beta and species of other genera with seeds of similar size;
100 g -	For bajra, jute and species of all other genera;
250 tubers / planting stakes/roots/corms -	Seed potato, sweet potato and other vegetatively propagating crops.



Basal pigmentation of seedlings in Pearl Millet



Seedling pigmentation in Wheat

Node pubescence



Node pigmentation



Internode pigmentation



CRITERIA FOR GOT TO DECIDE THE GENUINENESS OF VARIETY

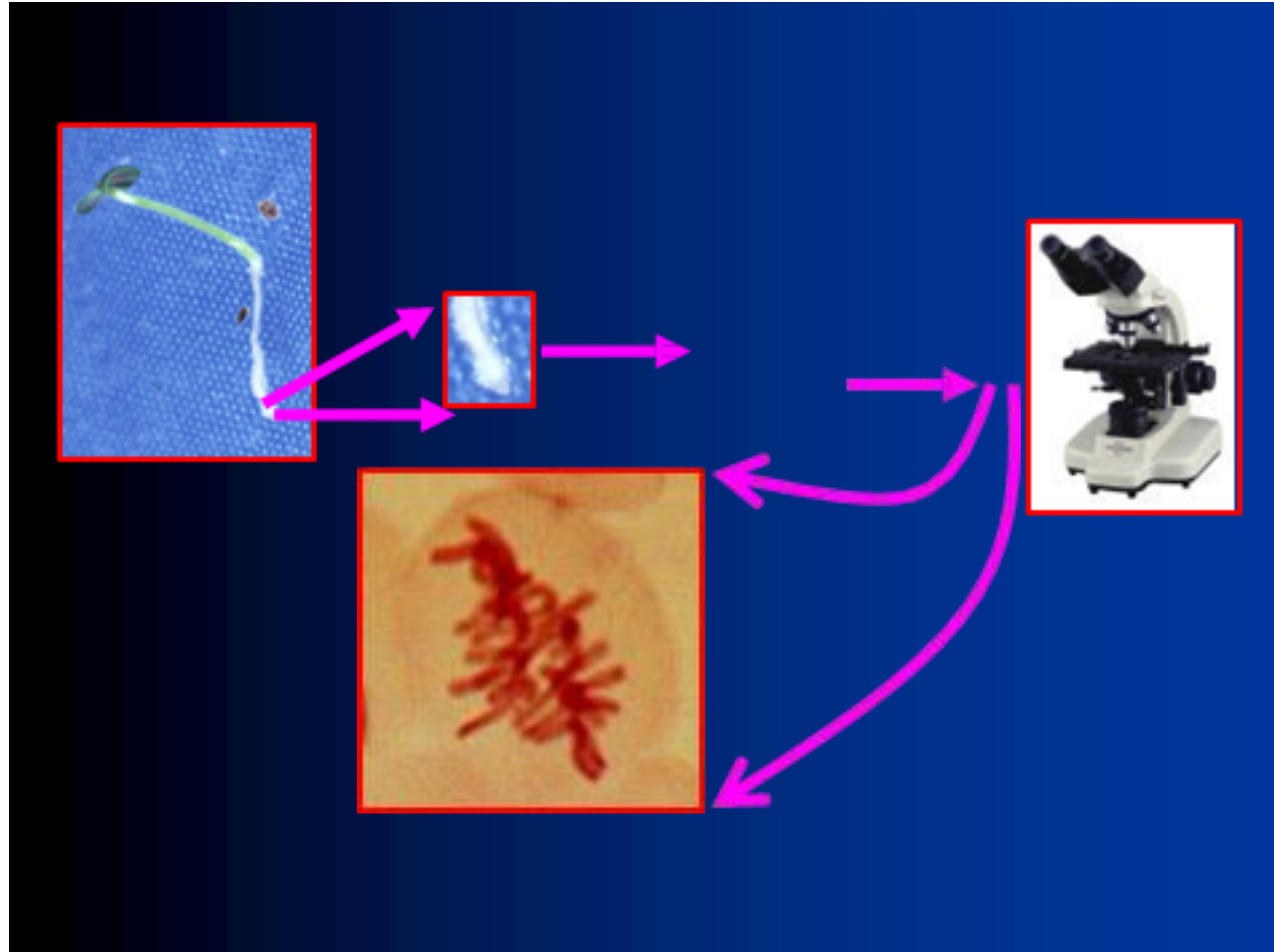
Maximum permissible off types (%)	Minimum genetic purity (%)	Number of plants required/sample for observation
0.10	99.9	4,000
0.20	99.8	2,000
0.30	99.7	1,350
0.50	99.5	800
1.00 and above	99.0 and below	400

REJECT NUMBER FOR PRESCRIBED STANDARDS AND SAMPLE SIZE

Standard	Reject number for sample size of	
	800	400
99.5(1 in 200)	8	*
99.0(1 in 100)	16	8
95.0(10 in 100)	48	24
90.0(10 in 100)	88	44
85.0(15 in 100)	128	64

*- indicates that the sample is too small for a valid test.

Cytological Test (Ploidy Test)



Drawbacks

- ▶ Time consuming Effect of environment
- ▶ Less number of plant morphological markers
- ▶ Environmental stress conditions often mask specific morphological traits.
- ▶ Large amount of land required.
- ▶ Laborious

Types of Chemical test

▶ ISTAAOSA

- ▶ Phenol test for wheat
- ▶ Lugol's test for lupin
- ▶ Electrophoretic technique for wheat, oat, peas, lolium, maize and sunflower
- ▶ Copper sulfate-ammonia test for sweet clover
- ▶ HCl test for oat
- ▶ Peroxidase test for soybean
- ▶ KOH test for rice and sorghum
- ▶ NaOH test for wheat

Phenol Test in Wheat

- ▶ Principle: The ‘**Tynosinase**’ enzyme present in the seed coat reacts with phenol vapours to No colour change produce melanin, a pigmented product.
- ▶ Based on enzyme activity the quantity of melanin produced varies and depending on which the seed colour varies from **colourless (no melanin production or no enzyme activity)**, **light brown (low enzyme activity)** to **black (high Dark Brown Light brown enzyme activity)**.
- ▶ Since enzyme activity is specific for cultivar, the colour reaction between cultivars will also vary aiding in varietal purity testing.



Peroxidase Test in Soybean

- ▶ **Principle:** The 'Peroxidase' enzyme present in the seed coat, oxidizes the 'Guaiacol' in the presence of H_2O_2 to impart reddish-brown colour
- ▶ **Drawback:** During reaction, the 'Guaiacol' may be oxidized by other oxidizing enzymes

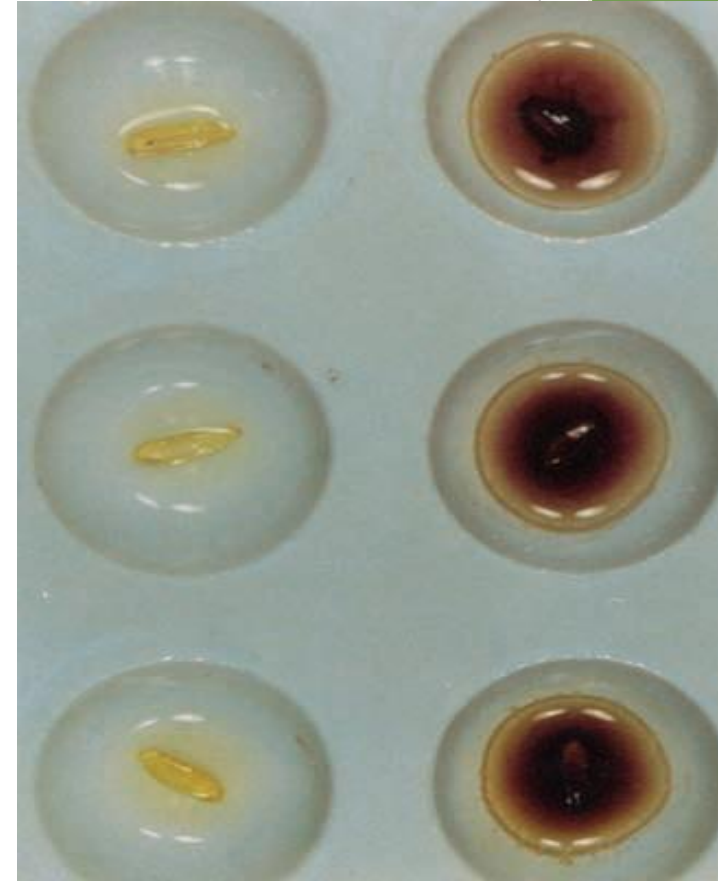


Copper Sulfate-Ammonia Test for Sweetclover (*Melilotus spp.*)



Potassium Hydroxide (KOH) Test for red rice (*Oryza sativa*)

- ▶ Due to the variation in emergence and maturity of red rice and the time required for the development of the red coloration on the grain, it is possible for colorless or nearly colorless seeds of red rice to contaminate rice seed lots.
- ▶ A simple KOH soak test for the detection of red rice seed was originally developed by Rosta (1975).



Sodium Hydroxide (NaOH) Test for Wheat (*Triticum aestivum*)



Demerits of Chemical Tests

- ▶ Difficult to distinguish the varieties falling in same group

Electrophoresis test

- ❖ Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
- ❖ Acid-page Alkaline-page
- ❖ Starch Gel Electrophoresis (SGE)
- ❖ Ultrathin Layer Isoelectric Focusing (UTLIEF)

Electrophoretic methods recommended by ISTA (1999) for various crops species

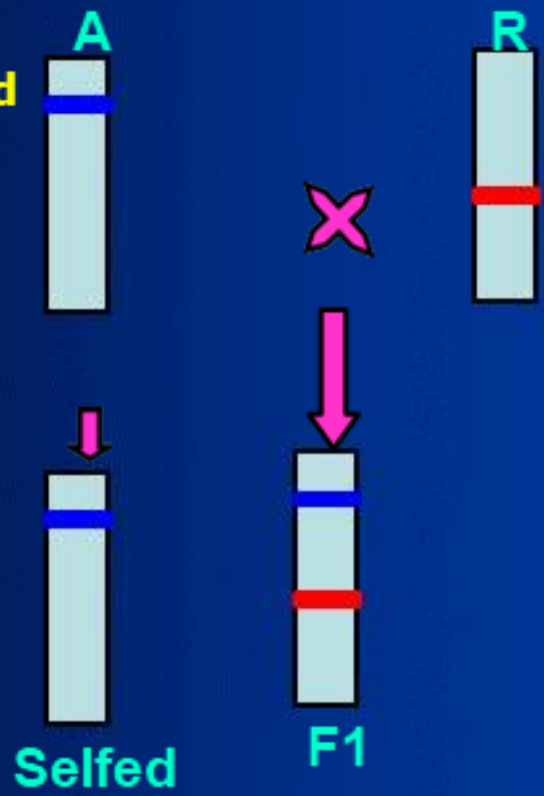
Crop species	Method
<i>Triticum and Hordeum</i>	Acid PAGE of alcohol soluble proteins (Gliadins from <i>Triticum</i> and Hordeins from <i>Hordeum</i>) using 100 individual seeds
<i>Pisum and Lolium</i>	SDS-PAGE of Tris-HCL soluble proteins using 100 individual seeds are used in <i>Pisum</i> while in <i>Lolium</i> bulked seeds are analysed. In <i>Lolium</i> the method will serve to varify seed lots but will not permit the detection of admixtures.
<i>Zea mays</i>	Alcohol soluble proteins (Zeins) extracted from individual seeds are separated by UTLIEF. Normally 200 single seeds analysed. For reports and issue of ISTA certificate, 400 single seeds are analysed.
<i>Avena sativa</i>	Urea/ethylene glycol-soluble proteins (Avenins) are extracted from seeds and separated by Acid-PGE

Evaluation

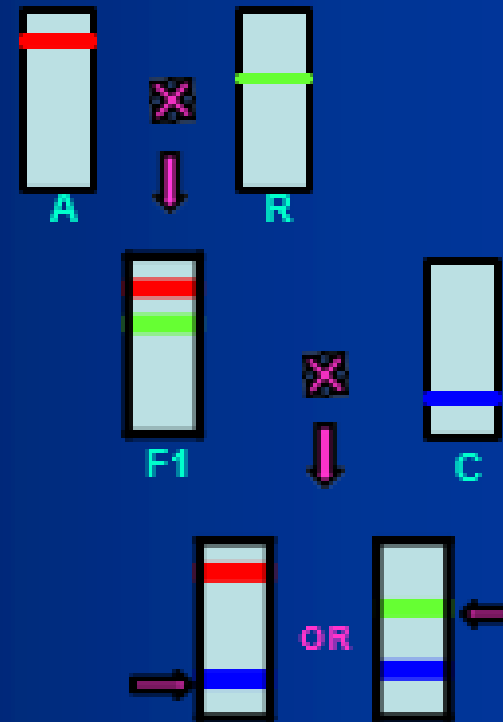
According to ISTA rules, verification of varieties is generally done by comparing the **electrophoretic profiles** of the **sample with that** of the authentic reference Variety with a well- described and established **protein banding pattern**.

In case of Hybrids, the protein patterns of the **hybrid was compared with the female and male parents**. Seeds with protein pattern similar to **female parent** are judged to be self pollinated. **Foreign pollinated seeds show a different pattern**.

Single cross hybrid

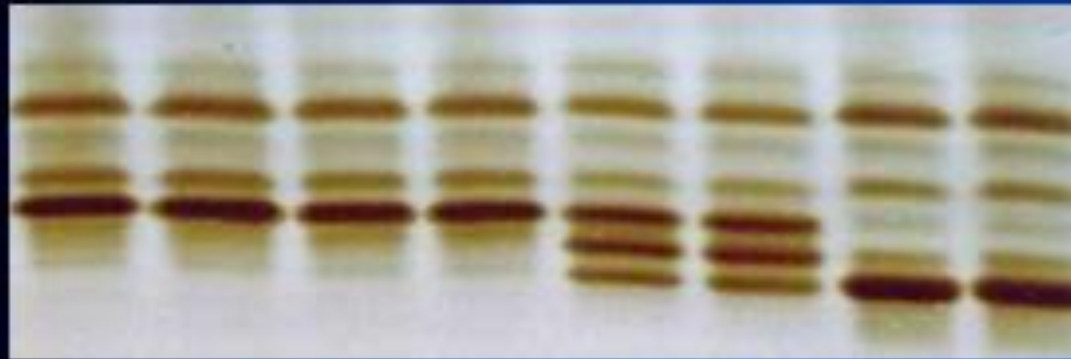


Three way cross Hybrid



Three way Hybrid

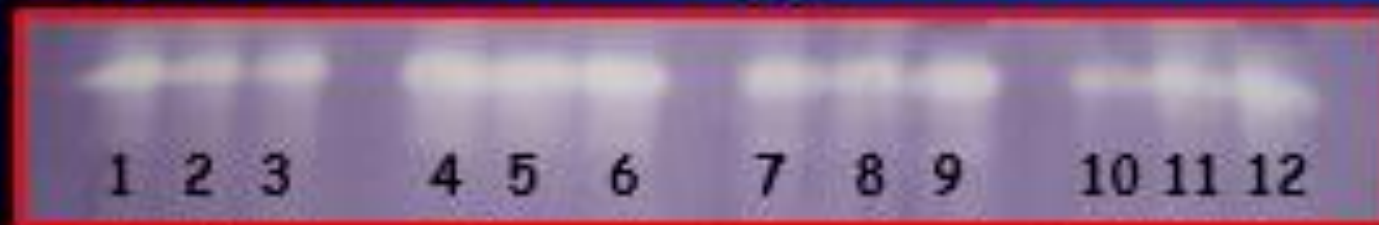
Electrophoretic profiles of seed esterases of Pearl millet hybrids and parental lines separated on Alkali-PAGE



Electrophoretic banding pattern of Seed Esterases



Electrophoretic banding pattern of SOD



1: ♂ Pusa Hybrid 5 4: ♂ Pusa Hybrid 6 7: ♂ ARBH 786 10: ♂ GBH2
2: ♀ Pusa Hybrid 5 5: ♀ Pusa Hybrid 6 8: ♀ ARBH786 11: ♀ GBH2
3: Pusa Hybrid 5 6: Pusa Hybrid 6 9: ARBH 786 12: GBH2

Comparative advantages and disadvantages of Polyacrylamide, SDS and IEF

	Polyacrylamide	SDS	IEF
Advantages	<ul style="list-style-type: none">■ Technical system well defined■ Excellent band resolution	<ul style="list-style-type: none">■ Technical system well defined■ Inexpensive■ Gels can be sliced	<ul style="list-style-type: none">■ Technical system well defined■ Uses charge rather than charge/density and size of proteins■ Gels can be blotted■ Short running time
Disadvantages	<ul style="list-style-type: none">■ Expensive■ Can not slice gel■ Potentially toxic	<ul style="list-style-type: none">■ Standardization of gels■ Long running time(5-6h)■ Poor band resolution	<ul style="list-style-type: none">■ Expensive■ Can not slice gel
IEF-Isoelectric Focusing			

Demerits

- ▶ **Stage specific**
- ▶ **Less number of plant morphological markers Less number of protein and isozymes markers**
- ▶ They are not affected by the field or greenhouse environment.
- ▶ They are cost effective compared to other methods and the turnaround time is relatively rapid.
- ▶ Multilocus analysis provide useful information for verifying inbred and hybrid genotypes.
- ▶ Most are co-dominant and many loci express at all stages of life cycle.
- ▶ An array of enzymatic analysis can be made using small quantities of leaf and seed material.
- ▶ There are limited number of marker isozymes as compared to molecular markers.
- ▶ **Stage specific expression of isozymes markers**

Need for Molecular Markers

- **Time consuming**
- **Requires large areas of land and skilled personnel**
- **Effect of environment**
- **Less number of plant morphological markers Less number of protein and isozymes markers Stage specific expression of isozymes markers**

Varietal Identification – Principle

- ▶ **Draw the representative submitted sample from seed lot**
- ▶ **Compare the DNA profile developed from seeds/seedlings/plants of prescribed size (working sample) obtained from the submitted sample with that of authentic sample of the variety**
- ▶ **Express the GP in % of seeds/seedlings/plants that are true to the type over the total used in test**

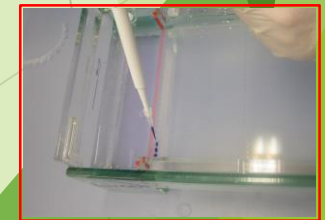
General methodology for molecular markers

- ▶ DNA extraction
- ▶ PCR amplification using nucleotide primer Initial Denaturation
- ▶ Repeated Cycles Denaturation Annealing Extension
- ▶ Final Extension
- ▶ Electrophoretic run and identification of PCR amplified product.

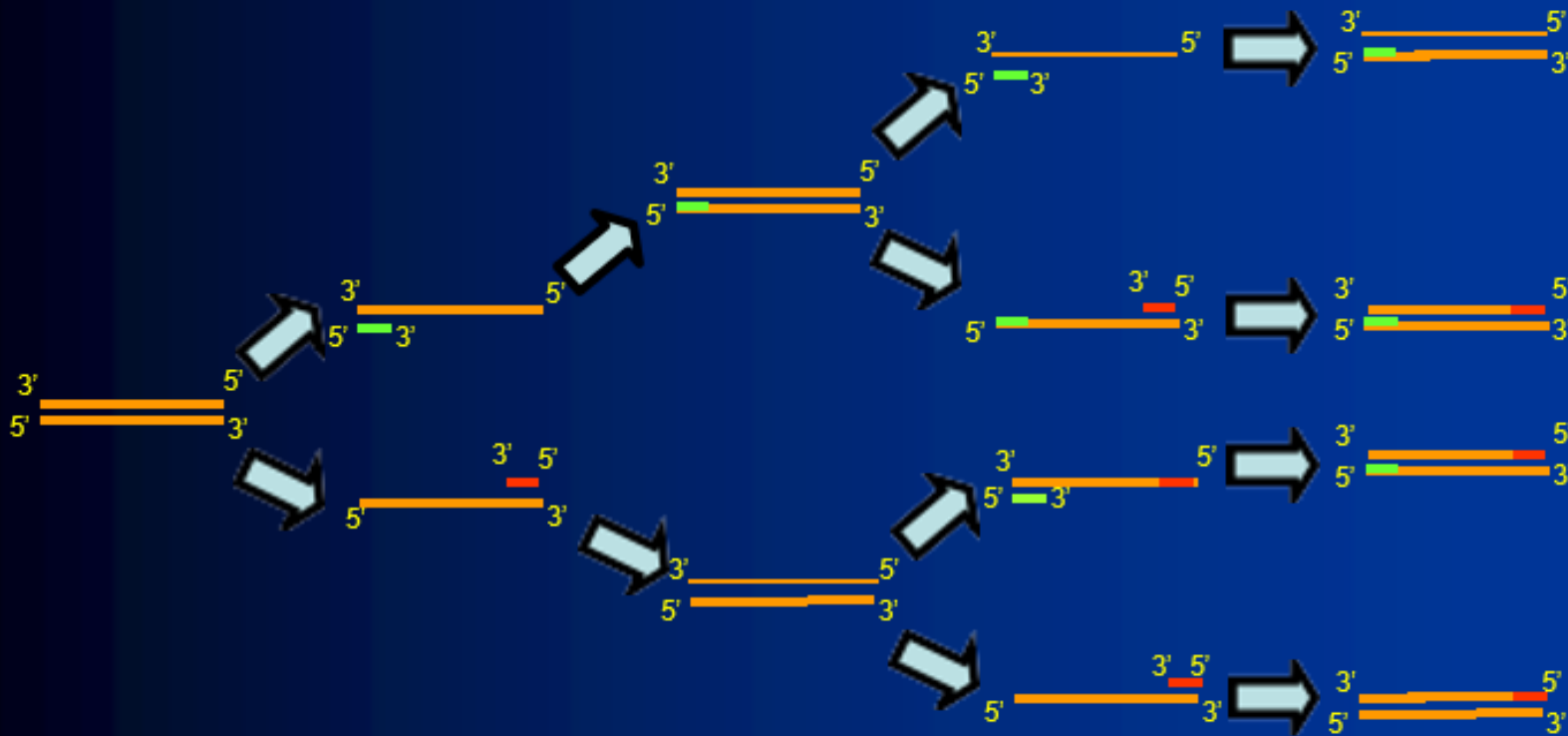
Developing DNA profile

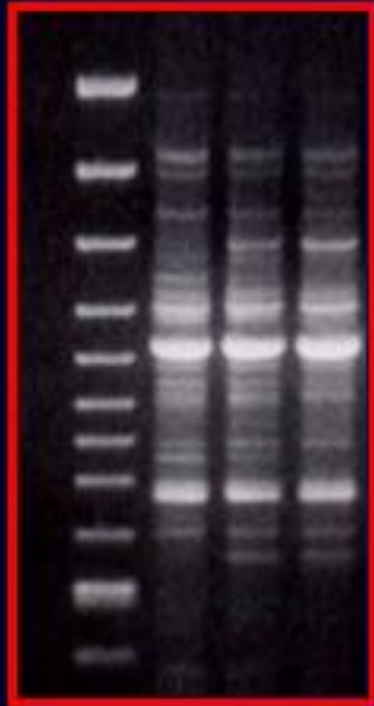
- ▶ DNA extraction from seeds/seedlings/plant tissues
- ▶ Generating the DNA fragments either by digestion of DNA with restriction enzymes (RFLP and AFLP) or by amplification of particular DNA fragment by PCR
- ▶ Separation of the DNA fragments in Agarose/poly Acrylamide medium

primers

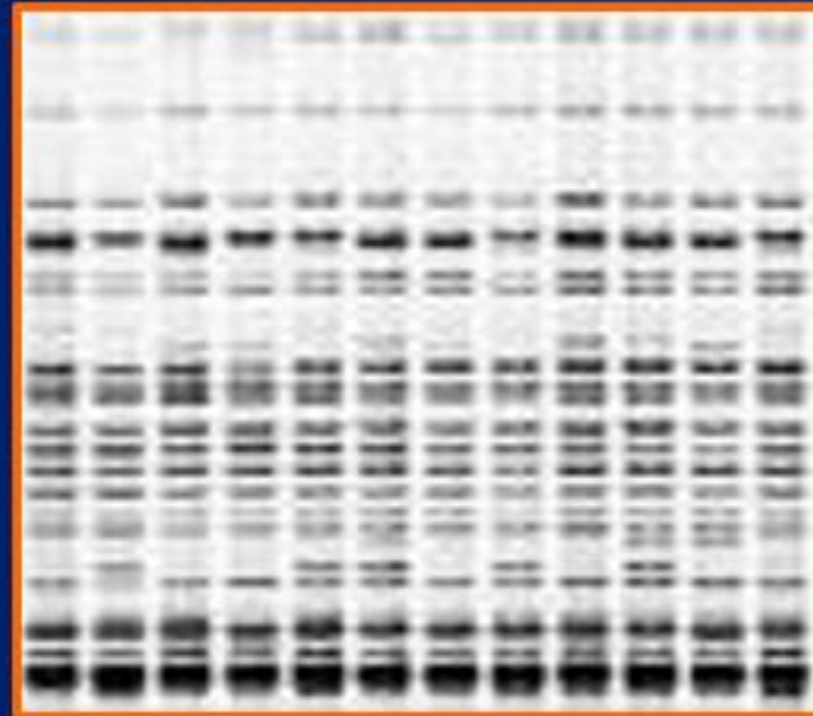


Polymerase Chain Reaction: Principle





Ethidium Bromide Stain



Auto Radiographic picture of the gel

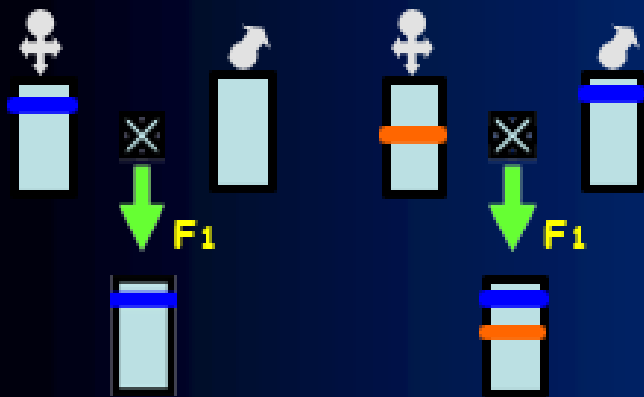
DNA Markers

Dominant Markers

- RAPD
- AFLP

Demerits:

Hybrids can not be distinguished from self seeds of parental lines

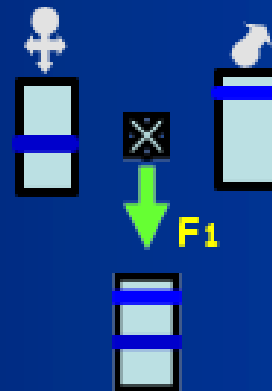


Co-Dominant Markers

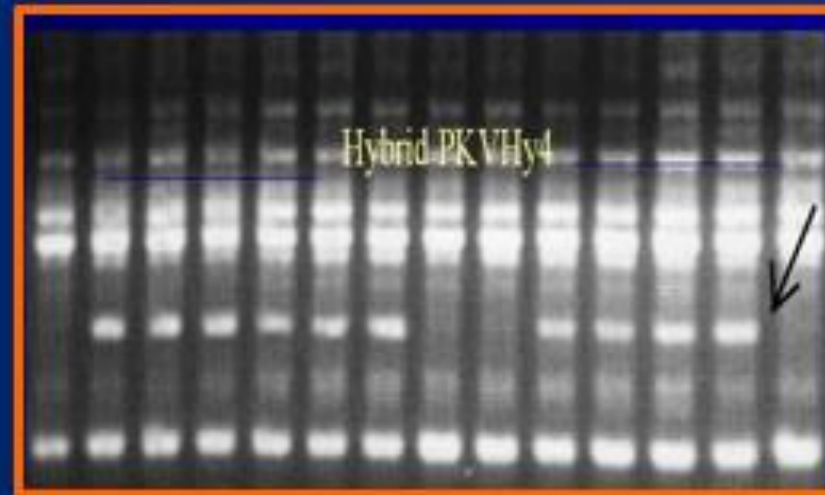
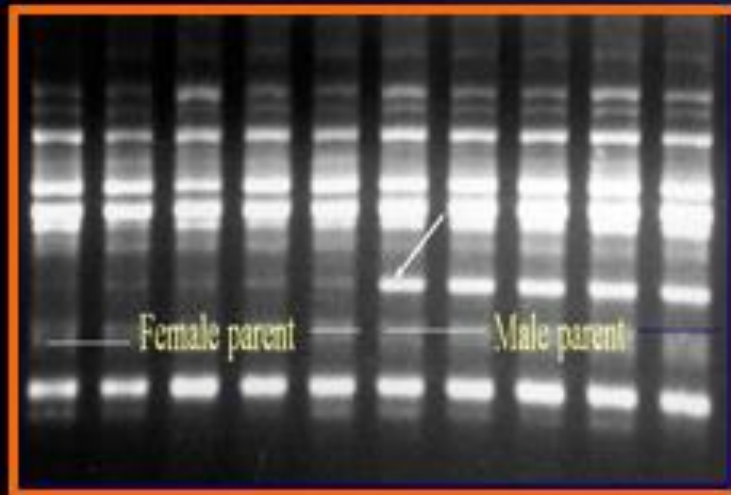
- RFLP
- SSR

Merits:

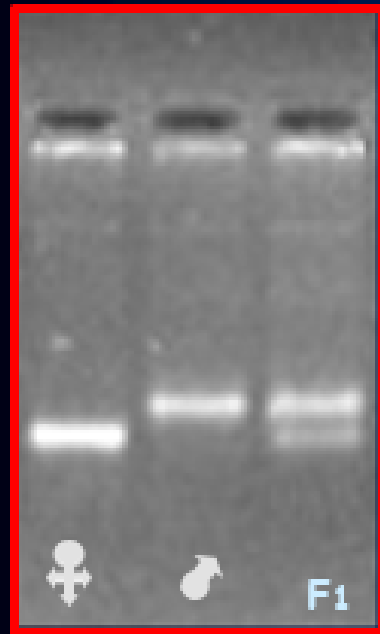
Hybrids can clearly be distinguished from self seeds of both the parents



Application of RAPD Marker to Assess the Purity of Cotton Hybrid Seed Lots



Application of SSR marker to test the purity of Rice hybrid PRH10

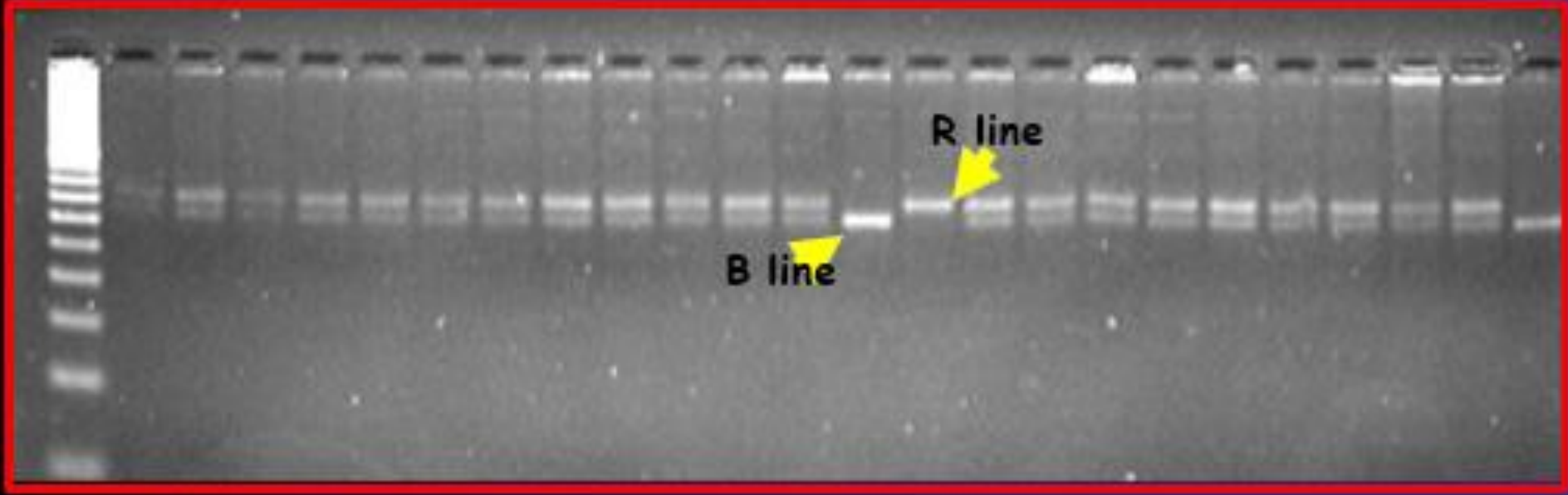


PUSA 6A

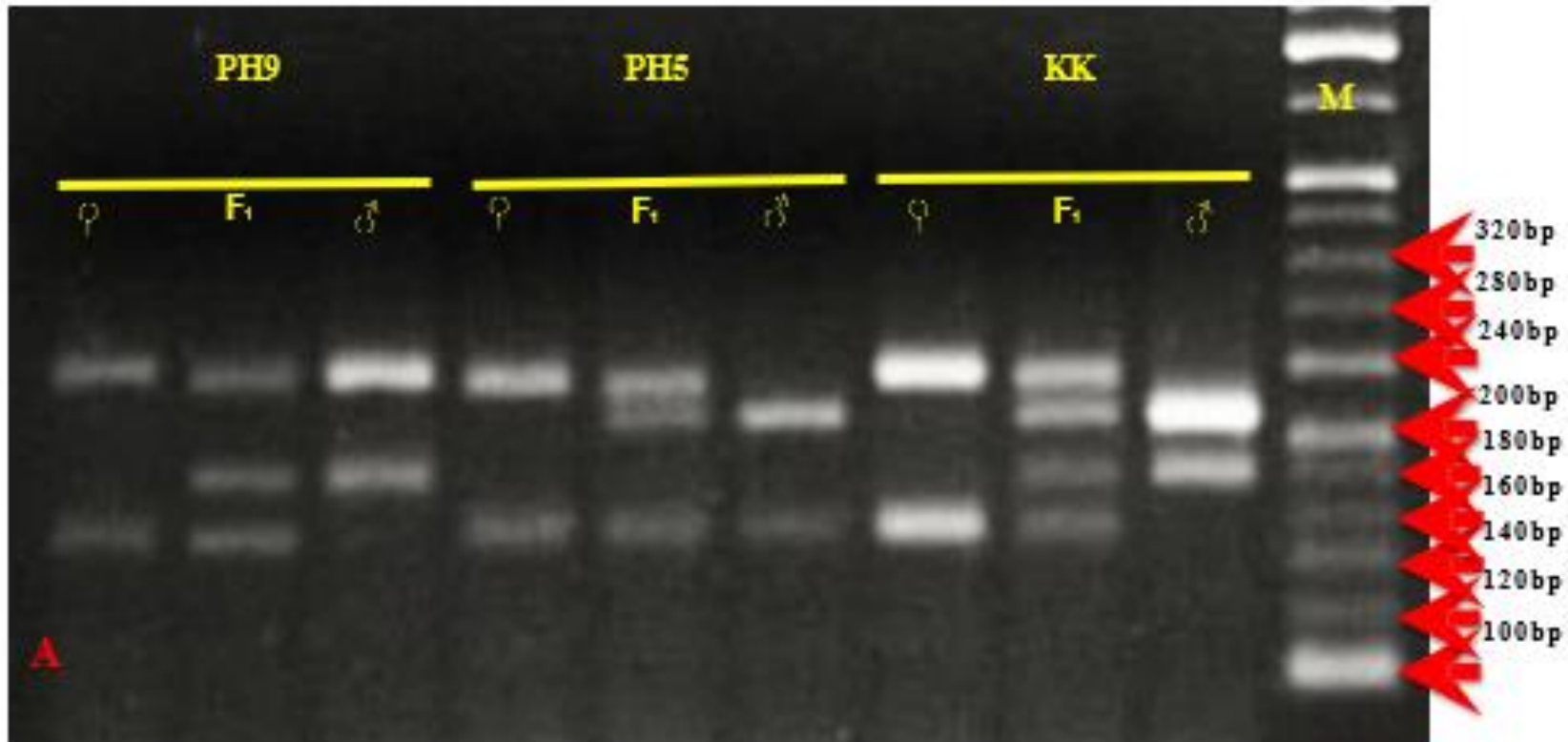
X

PRR 78

PRH 10



Application of SSR marker to establish the identity of Brinjal hybrids



Comparison of molecular techniques and their applicability

Characteristics	RFLPs	RAPDs	SSRs	AFLPs
Develop. costs (Rs. per probe)	Medium (4000)	Low (none)	High (20000)	Low (none)
Level of Polymorphism	Medium	Medium	High	Medium
Automation	No	Yes	Yes	Yes
Cost of Automation	High	Medium	High	High
Reliability	High	Low	High	Medium
Cost (Rs. per assay)	High (80)	Low (40)	Low (40)	Medium (60)
Radioactivity	Yes	No	Yes/No	Yes
Samples/day	20	50	50	50

Advantages of molecular techniques

- It has very large number of polymorphism development as compared to the bio-chemical markers.
- Residual heterozygosity can be detected.
- It is reliable to all crops.
- Very fast method

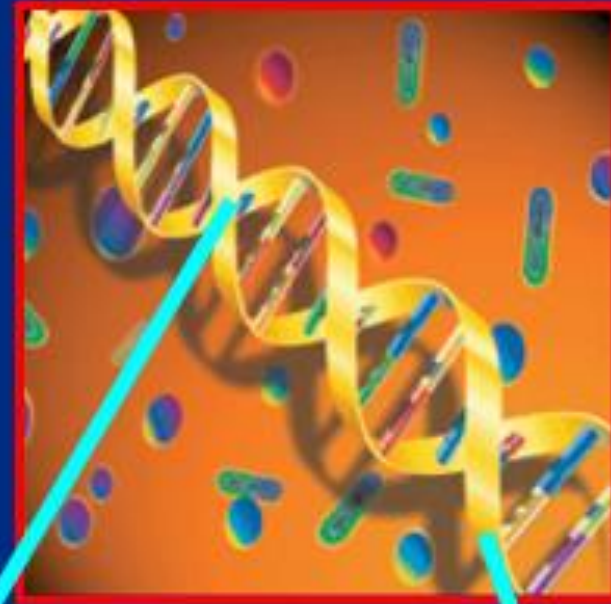
Limitations of molecular techniques

- Sophisticated instruments required.
- Very costly.

Testing varietal purity for transgenic crops

Transgenic Crop

The plant in which gene or genes from alien species are incorporated to improve certain economic traits like insect pest resistance, nutritive value of produce etc., is called Genetically Modified crop /Transgenic crop



GM crop detection - the rationale

- ▶ To ensure the **varietal purity** of GM seeds
- ▶ To **detect and quantify** the adventitious contamination
- ▶ To protect **the new GM crop** under PPV& FA
- ▶ To prevent the entry of **unapproved transgene**
- ▶ To certify that the seeds used in **organic farming** are free from GM seeds or its contamination

Criteria for fixing the thresholds limits

- ▶ Depends on the allowable content of GM contamination in the harvest
- ▶ Germany : Permitted adventitious GM
- ▶ material in food product is 1%
- ▶ 0.3% as threshold limit in seeds of cross pollinated crops
- ▶ 0.5% in case of self pollinated and vegetatively propagated crops

Phenotypic characterization (Herbicide Bioassays)

- ▶ Germination tests on solid germination media in the presence of a specific herbicide, where non-GM and GM seeds show distinct characteristics



Immunological Assays

🌐 Lateral Flow Sticks



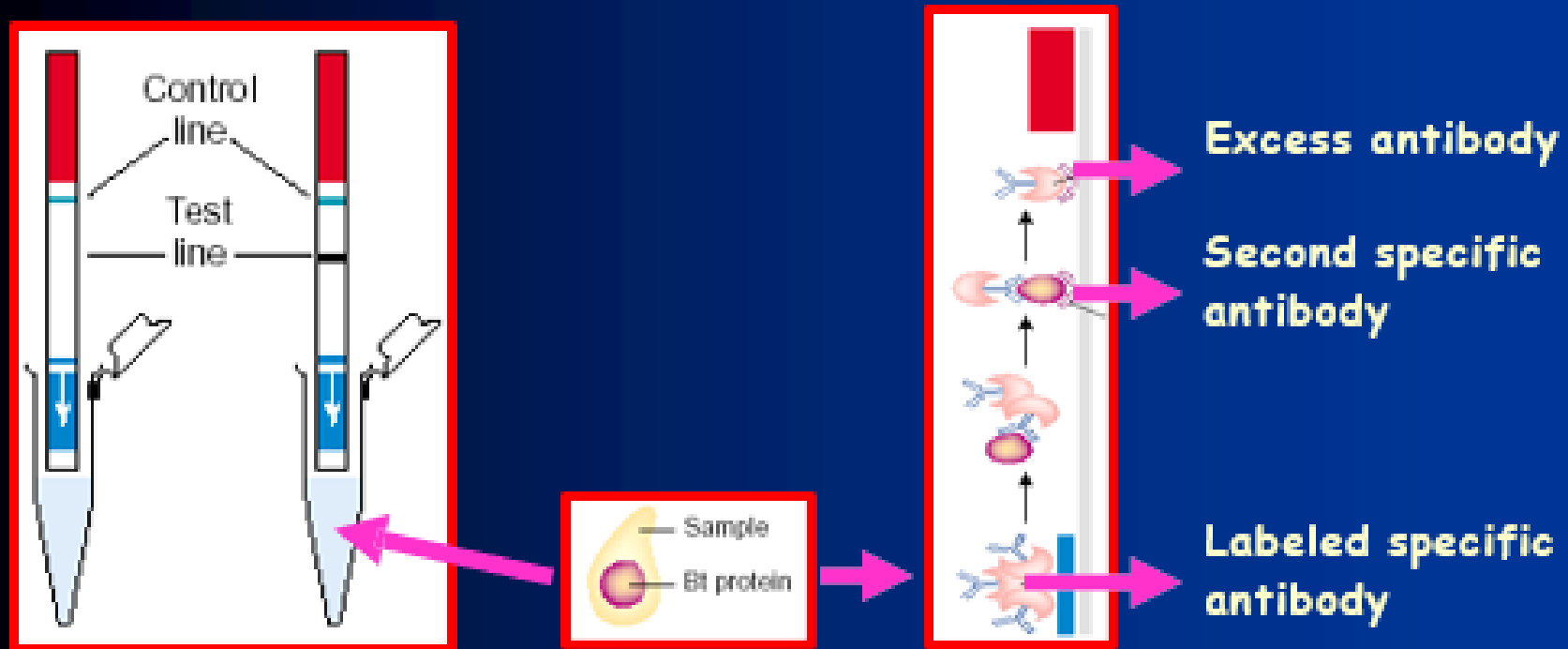
🌐 Enzyme Linked Immunosorbant Assay (ELISA)

Lateral Flow Strip Protocol



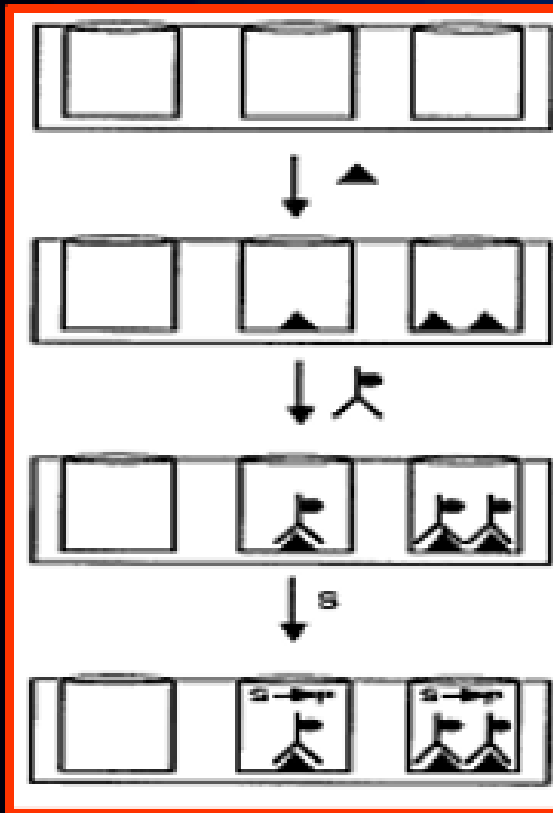
- 🌐 Punch leaf disc/seed
- 🌐 Add buffer and grind in tube
- 🌐 Insert flow strips for testing
- 🌐 Observe for band development

Lateral flow strips – the principle



- ELISA variation with immobilized double antibodies, specific to expressed proteins are coupled to a colour reactant and incorporated to a nitrocellulose strip

ELISA - Protocol



- Antigen added ELISA wells
- Enzyme-linked antibody added
- A colorless substrate added
- A colorless substrate is converted to a color product by the enzyme linked to antibody



Molecular detection of GMO

- ▶ PCR followed by electrophoresis
- ▶ Rt PCR