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11. Protocol for Estimation of Stigma Receptivity for Solving Sustainable Breeding Approaches

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11.1 Introduction:

To understand the theoretical aspect of natural sexual reproduction mechanism and practical exploitation of different concepts of breeding for improvement in crop production, measurement of time and duration of stigma receptiveness become necessary. Any success in breeding experiments or artificial pollination produce, should be accompanied by tests on the timing and duration of the stigma receptivity (Stone et al, 1995). The gynoecium is the counterpart of reproductive system act as a female partner in a plant. A gynoecium has carpel as its functional unit comprises of three parts a terminal stigma, sub-terminal style and basal ovule. The stigma is most essential part of any gynoecium where pollen shed for fertilization. Studies on stigma receptivity includes pollination experiments like hand pollination, seed set (Ramsey and Vaughton, 1991), morphological observations like simple microscopic study or SEM and TEM study (British and Berg, 1990, Fuss and Sedgley 1991 a, b) and study of enzymes and proteins present in stigma.

The pollen-pistil interaction involves adhesion of pollen grain on stigma, pollen tube growth and entry of pollen tube into ovule, all these processes depends on stigma receptivity (Shivanna et al, 1997). One of the crucial stage in the maturation of a flower which may greatly influence the rate of self-pollination, pollination success at different stages in the flower life cycle, the relative importance of various pollinators, the interference between male and female functions, the rate of competition via improper pollen transfer, and the chances of gametophytic selection (Galen et al., 1987). Receptive stigmas are characterized by high enzymatic activity (Makwana and Akarsh, 2017). The presence of several enzymes is found to coincide with this developmental stage (Knox, 1984; Shivana and Rangaswamy, 1992) and consequently most of the methods to determine stigma receptivity *in vitro* are based on the identification of enzymatic activity (Knox et al., 1986; Dafni 1992; Kearns and Inouye, 1993). The proteins and secretary fluids present at the stigma play a crucial role in pollen germination, pollen tube growth and successful fertilization (Heslop, 1975). The receptivity of stigmas are characterized by assaying the activity of several enzymes such as peroxidase, esterase, alcohol dehydrogenase, acid phosphatase and proteins studied in different species (Shivanna and Sastri, 1981).

The methods described in this chapter is based on the previous researcher's findings for estimation or knowledge or confirmation of stigma receptivity. It can be categorized into two types:

A. Qualitative determination of stigma receptivity
B. Quantitative determination of stigma receptivity

A. Qualitative Determination of Stigma Receptivity:

There are several methods of qualitative screening which indicates receptiveness of stigma in relation to days of a thesis and flower maturation.

These methods also help in proper localization and development of pollen tube on stigma. All observations done under low and high power of simple and compound microscope.

***a. In Vivo* Pollen Germination on stigma: (Joshirao and Saoji, 1989):**

Principle: Pollen grains germinate and form pollen tubes after they get deposited by the process of pollination on compatible stigma.

It emerges through one of the germ pore and passes through tissues of stigma and style to reach the ovule. The growing pollen tube is observed by staining with aniline blue.

Chemicals Required: absolute alcohol, glacial acetic acid, Sodium hydroxide, aniline blue, Disodium phosphate.

Procedure:

- Fix a part of stigma with some portion of style with acetic alcohol (1:1) in a small vial.
- Add few drops of 4N sodium hydroxide (NaOH) to soft stigma for 12-48 hours depending upon species.
- Wash the soft tissue with distilled water twice to remove the traces of sodium hydroxide.
- Stain the soft stigmas with aniline blue (water soluble aniline blue 0.05% in 0.05 M Disodium phosphate (Na_2HPO_4) on a slide, cover it with cover glass and press with thumb to make spreading of tissue into monolayer.
- Observe the slide under low (10X) and high (40X) power of compound microscope to count number of pollen germinate on stigma.

Observation Table:

Time	Total No. of stigmas observed	Mean No. of pollen retained on stigmas	Mean No. of germinated pollen	*In vivo* pollen germination (%)	Mean pollen tube length (μm)

b. Hydrogen Peroxide test for stigma receptivity: **(Kearns and Inouye, 1993)**

Principle: The peroxidase on the surface of stigma facilitates communication between pollen and pistil by loosening the cell-wall components of the stigma which allow the entry of pollen tubes to fertilize the ovule.

This solution contains hydrogen peroxide when in contact with peroxidases present on the stigmas creates a reaction.

When it is receptive promoting the formation of air bubbles that can be easily observed by the naked eye.

Chemicals Required: Hydrogen peroxide

Procedure:

- Take about 10 stigmas of different stage of flower development to evaluate the receptivity for each stage.
- The different stages viz., before a thesis (bud stage), full bloom and withering stage.
- Keep the stigma in a cavity slide and add two drops of 4% aqueous solution of hydrogen peroxide (H_2O_2)
- Observe the slides at 20 X magnification of simple microscope for amount of bubbles formed on the stigma surface.
- Record the observations in one hour time interval from 6.00 AM to 6.00 PM.

Observation Table:

Time	Total No. of stigmas observed	Mean No. of pollen retained on stigmas	Mean No. of germinated pollen	*In vivo* pollen germination (%)	Mean pollen tube length (μm)	Production of oxygen bubbles /minute

c. Baker's procedure of Alcohol Dehydrogenase based test (Galen and Plow right, 1987):

Principle: Alcohol Dehydrogenase (ADH) is an oxido-reductase coupled with NAD (P) H (reduced nicotinamide adenine dinucleotide phosphate) which require the continuous supply of molecular oxygen as an oxidant or electron acceptor.

The number of oxygen bubbles recorded on the stigma of flowers up to 7 days after the commencement of bloom stage, which indicates the degree of receptivity of stigma.

Chemicals Required: 1M phosphate buffer, nitro blue tetrazolium, nicotinamide adenine dinucleotide, Ethanol, hydrogen peroxide (H_2O_2)

Procedure:

- First prepare the test solution consist of 10 mL of 1M phosphate buffer (pH 7.4) diluted (1 part buffer to 2 part distilled water); 6 mg NBT (nitro blue tetrazolium); 5 mg NAD (nicotinamide adenine dinucleotide) and 1 mL ethanol.
- Cut the fresh stigma and put on large droplet of prepared test solution on a slide and incubate at 8 C in closed petridish containing moist filter paper in the bottom.
- Observe the stigma after 12–18 hrs under a microscope (X10) to locate the stained area.
- Place 6% of hydrogen peroxide (H_2O_2) solution on the stigma and observe the appearance of bubbles.

Observation Table:

Sr. no.	Flower stage	Stigma colour	Stigma type	Number of bubbles

d. Stigma receptivity by Benzidine-H_2O_2 test (Dafni and Maués, 1998):

Principle: A sensitive test with a solution of benzidine, hydrogen peroxide, and sulphuric acid. The stigma receptivity is the reaction of benzidine + hydrogen peroxide, which causes receptive stigmas to change from their natural color to dark blue, besides promoting the formation of easily observed air bubbles due to the action of the peroxidases contained in the hydrogen peroxide.

However, the method relying on the reaction of α-naphthyl-acetate with fast blue B salt is considered more reliable (albeit more expensive), because the action of this solution is based on the esterase reaction, avoiding false-positive results, by changing the stigma's natural color to a darker shade or black when receptive.

Chemicals Required: benzidine, hydrogen peroxide, and sulphuric acid paper bag

Procedure:

- Isolate the Florets in transparent sulfuric acid paper bag of 1 DA (day of anthesis) to 7 DAA (days after anthesis).
- Collect the pistils at 8:00 AM and detach the stigma from it.
- Treat stigma with benzidine-H_2O_2 solution (1% benzidine: 3% H_2O_2 hydrogen peroxide: water = 4:11:22, v/v) for 10 to 15 min at 28°C.
- The pistil was regarded as receptive when more than 2/3 of the stigmatic area were stained dark blue and associated with some amount of bubbles released.

Observation Table:

Sr. no.	Flower stage	Colour of stigma	Number of bubbles

e. Localization of estarses on stigma (Shivanna and Rangaswamy (1992)**:**

Principle: Esterases are important component of the stigma-surface protein and its presence related to stigma receptivity.

Non-specific estrases present on the cuticle of stigma may involve in breakdown of cuticle barrier detecting thin layer pellicle during pollen pistil interaction. This is quite instant method of detecting receptiveness of stigma.

Chemicals Required: Solution A contains fast blue B, sucrose (10% w/v), phosphate buffer (0.15M, pH 6.8). Solution B contains α-napthyl acetate.

Procedure:

- Dip the excised stigma with style of selected stages in solution 'A' and solution 'B' separately and incubated at 25^0C in a humidity chamber for 22 min.
- After the specific period of incubation (10-20 min), remove the stigmas from solution and wash with phosphate buffer (pH 6.8).
- Mount the treated stigmas in 50% glycerine and pressed with cover slip to make uniform layer.
- Observe under microscope for development of rust colour which indicate receptiveness of stigma as well as localization of estrases on stigma.

Observation Table:

Time	No. of germinated pollen on stigma	*In vivo* pollen germination (%)	Detection of estrases (+/-)	Intensity of stigma receptivity (Esterase activity)

Precautions for Qualitative estimation of stigma receptivity:

- All stigma were checked under a magnifier (X10) for any damage on stigmatic surface.
- Wash the tissue thoroughly after fixation unless traces of fixative can bleach the colour of stain.
- Proper temperature should maintain for performing experiments.
- The days and time of anthesis should properly note down.
- The observations should make for at least 10 samples for avoiding error and confirmation.
- Intensity of oxygen bubbles or responses towards stain should mention + and – sign.

B. Quantitative determination of stigma receptivity:

The different enzymes particularly estarses, catalases and peroxidises released during pollen germination on stigma. The quantitative measurement can done through UV-VIS spectrophotometer.

The receptive stigma-surface contains extra-cellular proteins either in the form of pellicle or as a component of exudates. Stigma-surface protein play a crucial role in pollen germination, pollen tube entry into the stigma and probably incompatibility responses. The stigma also covered with exudates containing lipids, phenolic compounds, carbohydrates, proteins, phosphates, lectins and amino acids including esterases.

a. Estimation of catalase enzyme activity of stigma (Kar and Choudhuri, 1987):

Principle: The UV light absorption of hydrogen peroxide solution can easily measure between 230-250 nm. When catalase decomposes H_2O_2 , the absorption decreases with time.

Chemicals required: Tris HCl, Titanium sulphate ($TiSO_4$), sulphuric acid (H_2SO_4), distilled water

Procedure:

- **Preparation of H_2O_2 solution:** Dissolve 0.025 ml of H_2O_2 in distilled water and adjust the final volume of that solution up to 50 ml by the addition of distilled water.
- **Preparation of Ti $(SO_4)_2$:** Dilute 15% stock solution of Ti $(SO_4)_2$ up to 0.8% by the addition of 25% H_2SO_4. For making 0.8% concentration of Ti $(SO_4)_2$; Mix 3.2 ml of Ti $(SO_4)_2$ stock solution with 56.8 ml of 25% H_2SO_4.
- Crush about 20 mg stigma tissue of each sample with 5000 µl Tris HCl extraction buffer in chilled clean mortar and pastel.
- Centrifuge each mixture at 5000 rpm for 10 minutes at 4° C. Collect supernatant as protein stock store in refrigerator at 0-4° C.
- Add 0.5 ml Hydrogen peroxide (H_2O_2) with equal amount of cold crude protein stock in an eppendorf tube.
- Incubate the reaction mixture at 37°C for 5 minutes Finally, terminate the reaction by adding of 1 ml 0.8% titanium sulphate [$Ti(SO_4)_2$]
- Prepare a blank set of by adding 1 ml Ti $(SO_4)_2$ with the reaction mixture before addition of H_2O_2.
- Observe the change of colour after 5 minutes.
- Centrifuge the reaction mixtures at 5000 rpm for 10 minutes.
- Collect Supernatant of each tube and measured the absorbance at 420 nm wavelength by using UV-VIS spectrophotometer.
- Calculate the enzyme activity using the formula of Fick and Qualset (1975).

b. Estimation of peroxidise enzyme activity of stigma (Biswas and Choudhuri, 1978)

Principle: Peroxidase (POD) includes a group of specific enzymes such as NAD-peroxidase and NADP-peroxidase.

Simply known as donar: H_2O_2-oxidoreductase. POD catalyses the dehydrogenation of large number of organic compound and its activities assayed spectrophotometrically at 430 nm.

Chemicals Required: phosphate buffer, monosodium dihydrogen phosphate, disodium hydrogen phosphate, pyrogallol, distilled water

Procedure:

- **Preparation of reaction buffer:**

Prepare 0.3 mM phosphate buffer at pH 6.8 for use as reaction buffer, by mixing the following chemicals.

a. Dissolve monosodium dihydrogen phosphate (NaH_2PO_4): 2.34 g in 50 ml of distilled water.
b. Dissolve disodium hydrogen phosphate (Na_2HPO_4): 2.112 g of in 50 ml of distilled water.
c. Finally, mix 25.5 ml of NaH_2PO_4 with 24.5 ml of Na_2HPO_4 solution and the final volume of this mixture adjust up to 100 ml with the addition of distilled water.

- **Preparation of pyrogallol solution:**

Dissolve 47.25 mg pyrogallol in 25 ml of distilled water and prepare this solution fresh before use.

- **Preparation of H_2O_2 solution:**

Dissolve 0.140 ml of H_2O_2 in distilled water and the final volume of that solution adjust 50 ml by the addition of distilled water.

- Mix 0.2 ml cold protein stock with 0.2 ml of reaction buffer (0.3 mM phosphate buffer at pH – 6.8). Also add.2 ml pyrogallol and 0.2 ml H_2O_2 with that reaction mixture.
- Incubate the reaction mixtures at 25°C for 2 minutes. After 2 minutes, terminate the reaction of each tube by adding of 0.2 ml 5% H_2SO_4.
- A blank set is prepared with 5% H_2SO_4 and incubate.
- Observe change in colour and measure absorbance at 430 nm wavelength using uv-vis spectrophotometer against the blank set.
- Calculate the enzyme activity using the formula of Fick and Qualset (1975).

Calculation of Enzyme Activity:

Determine the Catalase and Peroxidase activity by calculating the difference of absorbance (ΔA) between, the activity of enzyme and 'zero time' blank set following the formula. Enzyme activity was calculated in the form of unit/minute/g.

$$\text{Enzyme activity} = \frac{\Delta A \times T_V}{T \times V \times Wt}$$

Where,

TV = Total volume of protein extract

T = Total time of incubation

v = Volume of extract used in reaction mixture

Wt = Weight of the tissue from which enzyme was extracted

Observation Table:

Time	Catalase activity (unit/min/gm)	Peroxidase activity (unit/min/gm)

c. (A) Extraction of Total protein for stigma (Singh *et al*., 1993)

Chemicals Required: Tris HCl buffer

Procedure:

- Take 20 mg stigma samples.
- Homogenizes stigmas in 1500µl of 0.2 M Tris HCl buffer, pH 7.4 at 4°C.
- After that centrifuge at 10,000 rpm for 10 min. at 4°C.
- Collect the supernatant and store the stigma protein at -20°C for further analysis.
- Estimate the protein concentration by Bradford (1976) assay.

d. (B) Estimation of Total protein for stigma (Bradford, 1976)

Principle: The assay is based on the ability of proteins to bind with coomassie brilliant blue G 250 and form a complex whose extinction coefficient is much greater than that of dye.

Chemicals Required: BSA, Bradford reagent

Procedure:

- To prepare a blank set mix 1 ml Bradford reagent with 1 ml double distilled water.
- Add 10 µl of protein solution (previously extracted protein) with 990µl of double distilled water in a test tube.
- Add 1 ml of Bradford reagent to each test tube.
- Vortexed gently and incubated at 25°C for 10 minutes.
- The colour of the solutions on each tube turned blue.
- The absorbance was taken at 595 nm wavelength.
- Estimate amount of protein of each sample using the standard curve of Bovine Serum Albumin (BSA).
  Prepare 1 mg/ml Bovine Serum Albumin (BSA) stock as standard BSA solution and prepare standard curve using 0.01 ml, 0.02 ml, 0.04 ml, 0.06 ml, 0.08 ml and 1 ml of BSA stock solution against their respective optical density (OD) values at 595 nm.

The amount of total soluble protein present within the test plant samples can estimate using the equation: $\frac{\frac{Concentration of known}{protein sample of BSA}}{\frac{OD value at\ 595\ nm of known}{protein sample of BSA}} = \frac{\frac{Concentration of unknown}{protein of pollen}}{\frac{OD value at\ 595\ nm of unknown}{protein of pollen}}$

- From standard curve, concentration of known protein sample of BSA and O.D. value of BSA samples obtain and estimate amount of total soluble protein present in the stigma from the above equation.

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