Optimization of *in vitro* regeneration protocol

a. <u>With seedling derived explants</u>

Different explants derived from seedlings of 48-1 (Jwala) were tried on various media that were based on MS media combination supplemented with different hormone combinations. Basically four different seedling derived explants were tried and the major observations recorded are indicated below. A representative figure indicating the response is provided in Figure 1. As there was positive response with hypocotyl explants, many combinations of hormones were tried and the results are summarized in Table 1 and a representative picture of the response is indicated in Figure 2. Two media combinations were identified to be promising and they are highlighted below.

Major Observations

Shoot tips:

- Responded well with all the hormonal combinations.
- Multiple shoots (3-5) with BAP 0.5/1mg/l + TDZ 0.2/0.5 mg/l

Embryo axis: Responded giving single shoot and roots with BAP/2ip+IBA combinations

Cotyledons

- Green compact and friable callus with BAP+ IAA/IBA combination
- White compact callus with TDZ + IAA/IBA
- No shoot initiation observed in any hormonal combinations

Hypocotyls:

- Green compact callus with BAP+ IAA/IBA combination
- Green nodular callus with shoot initiation in **BAP+TDZ + IBA**
  - ✓ **1-2 mg/l 2ip+ 0.5 mg/l TDZ+ 0.2 mg/l IBA,**
  - ✓ **0.5 mg/l BAP+ 0.5 mg/l TDZ+ 0.1 mg/l IBA,**
  - ✓ 1 mg/l BAP+ 0.5 mg/l TDZ+ 0.5 mg/l IBA,
  - ✓ 1.5 TDZ+0.5 BAP+ 0.75 IBA

Table 1. Response of hypocotyl explants on different media for direct/callus mediated regeneration

Media	**Response**	**Media**	**Response**
TDZ 0.05 mg/l	No response	BAP 0.1 mg/l + 0.2 mg/l IAA	No response
TDZ 0.1 mg/l+ 0.1 mg/l IAA	Green compact callus formed	BAP 0.2 mg/l + 0.5 mg/l IAA	No response

TDZ 0.2 mg/l+ 0.1 mg/l IAA	Green compact callus formed	BAP 0.5 mg/l +0.5 mg/l IAA	Green nodular callus and shoot initiation observed
TDZ 0.5 mg/l+0.5 mg/l IBA	Green compact nodular callus formed	BAP 1.0 mg/l + 0.5mg IAA	Green compact callus
TDZ 1mg/l +0.5 mg/l IBA	White friable nodular callus formed	BAP 0.1 mg/l + 0.2 mg/l IBA	white callus observed
TDZ 2mg/l +0.5 mg/l IBA	White friable nodular callus formed	BAP 0.2 mg/l + 0.5 mg/l IBA	white callus observed
TDZ 1.5 mg/l +0.5 BAP+ 0.75 IBA	Multiple shoot initiation observed	BAP 0.5 mg/l +0.5 mg/l IBA	Green nodular callus and shoot initiation observed
TDZ 2mg/l +0.5 BAP+ 0.75 IBA	White friable nodular callus formed	BAP 1.0 mg/l + 0.5mg IBA	Green nodular callus and shoot initiation observed
TDZ 3mg/l +0.5 BAP+ 0.75 IBA	White friable nodular callus formed	BAP 0.1 mg/l +0.5 mg/l TDZ + 0.2 mg/l IAA	white callus observed
TDZ 1.5 mg/l +0.5 BAP+ 0.5 mg/l IBA	White friable nodular callus formed	BAP 0.2 mg/l + 0.5 mg/l TDZ + 0.5 mg/l IAA	white callus observed
TDZ 2 mg/l +0.5 BAP+ 0.5 mg/l IBA	White friable nodular callus formed	BAP 0.5 mg/l +0.5 mg/l TDZ + 0.5 mg/l IAA	Multiple shoots initiation observed
TDZ 3mg/l +0.5 BAP+ 0.5 mg/l IBA	White friable nodular callus formed	BAP 1.0 mg/l +0.5 mg/l TDZ + 0.5 mg/l IAA	Multiple shoots initiation observed
BAP 1.0 mg/l + 0.5mg IAA	white callus observed	2 ip 3mg/l + 0.5 mg/l TDZ + 0.5 mg/l IBA	contaminated
BAP 0.1 mg/l + 0.2 mg/l IBA	white callus observed	BAP 0.1 mg/l + 0.2 mg/l NAA	No response
BAP 0.2 mg/l + 0.5 mg/l IBA	white callus observed	BAP 0.2 mg/l + 0.5 mg/l NAA	No response
BAP 0.5 mg/l +0.5 mg/l IBA	Green callus observed	BAP 0.5 mg/l +0.5 mg/l NAA	Green nodular callus and shoot initation observed
BAP 1.0 mg/l + 0.5mg IBA	Green callus observed	BAP 1.0 mg/l + 0.5mg NAA	Green compact callus
BAP 0.1 mg/l +0.5 mg/l TDZ + 0.2 mg/l IBA	white callus observed	BAP 0.5 mg/l +0.8 mg/l NAA	Green nodular callus and shoot initation observed

BAP 0.2 mg/l + 0.5 mg/l TDZ + 0.5 mg/l IBA	white callus observed	BAP 0.1 mg/l + 0.2 mg/l 2,4-D	White callus observed
BAP 0.5 mg/l +0.5 mg/l TDZ + 0.5 mg/l IBA	Multiple shoots observed	BAP 0.2 mg/l + 0.5 mg/l 2,4-D	White callus observed
2ip 0.1 mg/l +0.5 mg/l TDZ + 0.2 mg/l IBA	Green nodular callus observed	BAP 0.5 mg/l +0.5 mg/l 2,4-D	Green nodular callus and shoot initation
2 ip 0.5 mg/l + 0.5 mg/l TDZ + 0.5 mg/l IBA	Green nodular callus observed	BAP 1.0 mg/l + 0.5mg 2,4-D	Green compact callus
2 ip 0.8mg/l + 0.5 mg/l TDZ + 0.5 mg/l IBA	Green nodular callus observed	2,4-D 0.2mg/l	White callus observed
2 ip 1 mg/l +0.5 mg/l TDZ + 0.2 mg/l IBA	Multiple shoots observed	2,4-D 0.5 mg/l	White callus observed
2 ip 2 mg/l +0.5 mg/l TDZ + 0.5 mg/l IBA	Multiple shoots observed	2,4-D 1.0 mg/l	White callus observed
BAP 1.0 mg/l + 0.5mg IAA + 0.1 mg/l IBA	Green callus observed	2 ip 3 mg/l +0.5 mg/l BAP + 0.5 mg/l IBA	Green nodular callus
BAP 0.1 mg/l + 0.2 mg/l IBA + 0.1 mg/l IBA	white callus observed	BAP 0.1 mg/l + 0.1 mg/l TDZ + 0.2 mg/l NAA	white callus
BAP 0.2 mg/l + 0.5 mg/l IBA+ 0.1 mg/l IBA	white callus observed	BAP 0.2 mg/l + 0.1 mg/l TDZ + 0.5 mg/l NAA	White callus
BAP 0.5 mg/l +0.5 mg/l IBA +0.1 mg/l IBA	Green callus observed	BAP 0.5 mg/l +0.1 mg/l TDZ + 0.5 mg/l NAA	Green nodular callus and shoot initation observed
BAP 1.0 mg/l + 0.5mg IBA+0.1 mg/l IAA	Green callus observed	BAP 1.0 mg/l + 0.1 mg/l TDZ + 0.5mg NAA	Green compact callus observed
BAP 0.1 mg/l +0.5 mg/l TDZ + 0.2 mg/l IAA	white callus observed	BAP 0.5 mg/l +0.12mg/l TDZ + 0.8 mg/l NAA	Green compact callus observed
BAP 0.2 mg/l + 0.5 mg/l TDZ + 0.5 mg/l IAA	white callus observed	BAP 0.1 mg/l + 0.2 mg/l TDZ + 0.2 mg/l 2,4-D	Green compact callus observed
2ip 0.1 mg/l +0.5 mg/l BAP + 0.2 mg/l IBA	Green observed nodular callus	BAP 0.1 mg/l + 0.5 mg/l TDZ + 0.2 mg/l NAA	white callus
2 ip 0.5 mg/l + 0.5 mg/l BAP + 0.5 mg/l IBA	Green nodular callus observed	BAP 0.2 mg/l + 0.5 mg/l TDZ + 0.5 mg/l NAA	White callus

2 ip 0.8mg/l + 0.5 mg/l BAP + 0.5 mg/l IBA	Green nodular callus observed	BAP 0.5 mg/l +0.5mg/l TDZ + 0.5 mg/l NAA	Green nodular callus and shoot initation observed
2 ip 1 mg/l +0.5 mg/l BAP + 0.2 mg/l IBA	Green nodular callus and multiple shoot initation observed	BAP 1.0 mg/l + 0.5 mg/l TDZ + 0.5mg NAA	Green nodular callus and shoot initation observed
2 ip 2 mg/l +0.5 mg/l BAP + 0.5 mg/l IBA	Green nodular callus observed	BAP 0.5 mg/l +0.12mg/l TDZ + 0.8 mg/l NAA	Green compact callus observed

b. <u>With explants derived from pre-treated embryos</u>

Mature embryos from cultivar 48-1 (Jwala) were pretreated with TDZ (0.3 mg/l) for 5-6 days and then hypocotyl explants derived from them were inoculated on MS medium supplemented with (2 Mg/l) 2-iP + (0.5 Mg/l) IAA + (0.1 Mg/l) TDZ and incubated for 15 days. Subsequently they were subcultured on to MS medium supplemented with (1 Mg/l) 2-iP + (1.0 Mg/l) BA+ (0.5 Mg/l) TDZ + (0.5 Mg/l) IBA and incubated for next 15 days. Later the explants with shoot initiation were subcultured on to MS medium supplemented with (0.5 Mg/l) BA – for shoot elongation. This combination gave promising results with more that 60 % (131/200) explants showing some nodular structures on the first medium, about 65 explants showing shoot initials in second medium and about 60 shoots appearing in 3rd medium. This combination is being repeated now with more number of explants . Some representative pictures are given in Figure 3 to indicate the kind of response seen with the explants on the three media combinations.

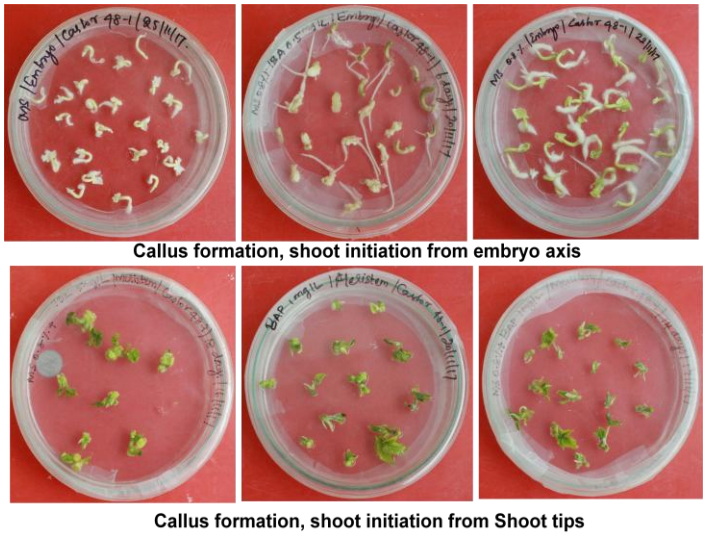

Fig 1. Response seen with embryo axis and shoot tips derived from seedlings

Callus formation, shoot initiation from hypocotyls

Fig 2. Response observed with hypocotyl explants on the promising media combinations

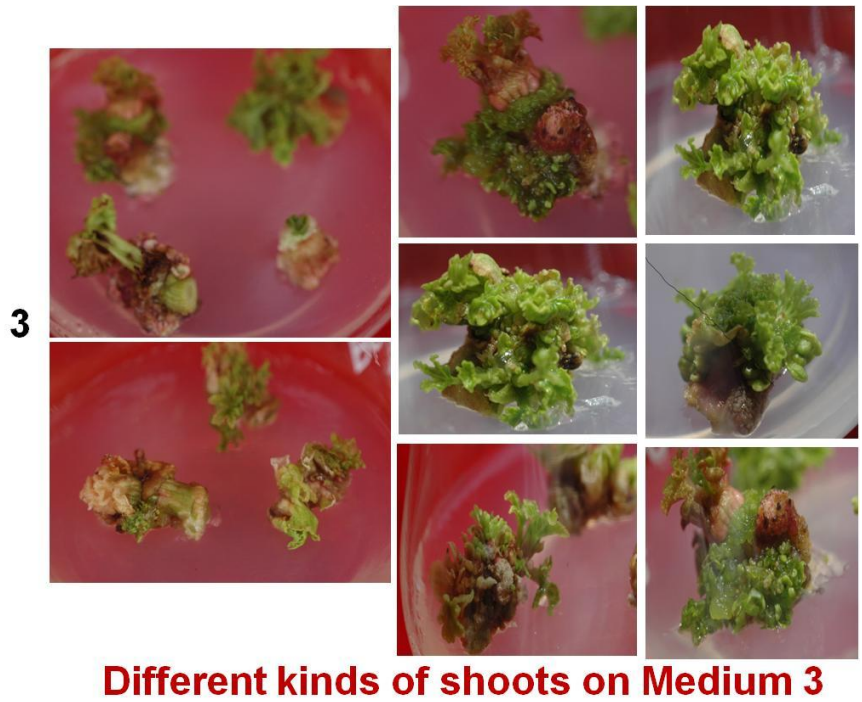

Figure 3. Response seen with explants derived from pre-treated embryos on different media. The three media are indicated on the sides.

1: MS+2 mg/l 2-iP + 0.5 mg/l IAA + 0.1 mg/l TDZ
2. MS+1 mg/l 2-iP + 1.0 mg/l BA+ 0.5 mg/l TDZ + 0.5 mg/l IBA
3. MS + 0.5 mg/l BAP

In summary, during the *in vitro* studies

- ✓ More than 100 media combinations were tried with hypocotyl and shoot apex as explants
- ✓ Explants subjected to with or without pre-treatment (with 0.3 mg/l TDZ)
- ✓ Some media combinations that initiate shoot with hypocotyl explants identified
- ✓ They are being tested now with more number of explants and replications