ICAR PUBLICATIONS

ANNUAL PROGRESS REPORT OF AD HOC PROJECT

"*In vivo* and *in vitro* mutagenesis for inducing *Fusarium* wilt resistance in pea (*Pisum sativum* L.)"

F.No. 8 (30) 03 Hort. II

Report Period: Ist January, 2005 to 31st December, 2005

Report No. : First

Principal Investigator: **Dr. Akhilesh Sharma**
Assistant Professor (Vegetables)

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Palampur- 176 062 (H.P.)

ANNUAL PROGRESS REPORT OF THE ICAR SPONSORED *AD HOC* SCHEME

1.	**Project Title**	***In vivo* and *in vitro* mutagenesis for inducing *Fusarium* wilt resistance in pea (*Pisum sativum* L.)**
2.	**Sanction No.**	8 (30) 03 Hort. II dated 17-11-2004.
3.	**Date of Start**	Ist January, 2005
4.	**Date of termination**	31st December, 2007.
5.	**Institution's**	
	Name	CSK HPKV, Palampur
	Place	Palampur
	Distict	Kangra
	State	Himachal Pradesh
	Dept. /Div. Name	Vegetable Science and Floriculture
	Actual location of research scheme to be carried out	Department of Vegetable Science & Floriculture, CSKHPKV, Palampur and Advanced Centre for Hill Bio-Resources and Bio-technology, CSKHPKV, Palampur.
6.	**Principal Investigator**	
	Name	**Dr. Akhilesh Sharma**
	Designation	Assistant Professor (Veg.)
	Div./ Section	Vegetable Science & Floriculture, College of Agriculture, CSKHPKV, Palampur- 176 062.
	Experience	9 years and 4 months
	Address	Vegetable Science & Floriculture, College of Agriculture, CSKHPKV, Palampur- 176 062
7.	**Co-Principal Investigators**	**I. Dr. Prikshit Plaha**, Professor Advanced Centre for Hill Bio-Resources and Bio-technology, CSKHPKV, Palampur. **II. Dr.Rajeev Rathour**, Assistant Scientist Advanced Centre for Hill Bio-Resources and Bio-technology, CSKHPKV, Palampur.
8.	**Objectives :**	1. Induction of *Fusarium* wilt resistant mutants in pea using mutagens. 2. Induction and isolation of *in vitro* induced mutants, 3. Evaluation of *in vivo* and *in vitro* induced mutants for disease resistance and agronomic performance.

9. Technical Programme as approved for the scheme

First year:

- Exposure of seeds of susceptible cultivars Azad P1 (main season Variety) and Arkel (early variety) to gamma rays (15 and 20 KR) in Co^{60} gamma cells.
- Treatment of seeds with 0.2 and 0.3 per cent ethyl methane sulphonate (EMS)
- Growing of treated seeds (M_1generation) and harvesting individual plants.
- Using appropriate explants (embryo and nodal segment) to develop calli.
- The calli will be transferred to solid media containing culture filtrate/ toxin of the wilt pathogen (*F. oxysporum* f.sp. *pisi)*.

Second Year

- Raising plant-to-row progenies (M_2 generation; around 2000 plants) in sick plots.
  *(Sick plots, which have a very long history of growing wilt susceptible pea cultivars, already exist in the Vegetable farm of the University. To avoid escape, these plots will also be inoculated with the *in vitro* multiplied inoculum of the virulent isolates of *F. oxysporum* f.sp. *pisi*. We have already identified the races of wilt pathogen prevalent in Himachal Pradesh in a recently concluded ICAR funded *ad hoc* project entitled "Molecular identification of *F. oxysporum* f.sp. *pisi* and characterization of its variability". The inoculum of these races will be incorporated in the field as well as pots for selecting durable wilt resistant mutants)
- Identification of wilt resistant plants and harvest them.
- These resistant plants will be subsequently confirmed under laboratory conditions.
- The toxin resistant calli will be transferred to regeneration media.
- The putative toxin resistant regenerants will be screened in Fusarium sick plots to identify resistant regenerants.
- Resistant plants will be harvested.

- Growing of irradiated and EMS treated seeds (M_1 generation) to get more disease resistant mutants.

Third Year

- The resistant mutants obtained through *in vivo* mutagenesis and *in vitro* selection will be further confirmed and inheritance will be established.
- The mutants will be evaluated for yield and other horticultural traits.

Raising M_2 progenies of the material raised during the second year. The resultant mutants obtained will further be confirmed. (This material will be further evaluated after the completion of the project to select wilt resistant and horticulturally superior garden pea lines).

10.	**Duration of the scheme**	**3 years (2005-07)**
11.	**Total cost of the scheme (Details as under)**	**Rs. 16,62,600/- (Rs, Sixteen lakhs sixty two thousands and six hundred only)**

Sr. No.	Description	No. of Posts	1st year	2nd year	3rd year	Total
a.	Senior Research Fellow	2	1,92,000	1,92,000	2,16,000	6,00,000
	HRA		6,000	6,000	6,600	18,600
b.	Recurring contingency (including TA)		1,80,000	1,80,000	1,80,000	5,40,000
	Total		**3,78,000**	**3,78,000**	**4,02,600**	**11,58,600**
c.	10% Institutional charges*		18,000	18,000	18,000	54,000
d.	Non-recurring contingencies		4,50,000	-	-	4,50,000
i.	**Poly house (i)**		**1,00,000**			
ii.	**Renovation of exiting lab facilities**		**1,00,000**			
iii.	**Repair of exiting green houses**		**1,00,000**			
iv.	**- 20°c Freezer**		**1,50,000**			
	Grand Total		**8,46,000**	**3,96,000**	**4,20,600**	**16,62,600**

12.	**Total amount sanctioned of the scheme**	Rs. 16,62,600/-
13.	**Total amount spent (01.01.2005 to 31.12.2005) as per details given below**	**Rs. 6,26,657/- + Rs. 92250/-***

Description	**Amount allocated (Rs.)**	**Amount Spent (Rs.)**	**Balance Amount (Rs.)**	**Remarks**
Salaries	1,98,000	1,05,673	92,327	Money was allocated during May,2005 as a result appointments were delayed
Recurring contingency	1,80,000	1,79,834	166	-
10% institutional charges	18,000	18,000	-	-
Poly house	1,00,000	1,00,000	-	-
Renovation of existing green houses	1,00,000	7,750	92,250*	*Case sent for approval to the Central Purchase Committee of the University and work will be accomplished as and when approval will be granted.
-20^{0}C Deep Freezer	1,50,000	1,13,400	36,600	The balance amount may kindly be allocated to purchase digital camera which will be of immense importance to get prints of different mutants.
Renovation of existing Laboratory facilities	1,00,000	1,00,000	-	-
Total	**8,46,000**	**6,24657**	**1,29,093+ 92,250***	

14.	Result of Practical/ scientific value	The project work is in the beginning stage
15.	Paper published	None

16. Detailed Report

In vivo:

- During the first year, seeds of *Fusarium* wilt susceptible pea cultivars Azad P-1 (main season variety) and Arkel (early variety) were exposed to γ- rays (15 and 20 KR) in CO^{60} gamma cells at Nuclear Research Laboratory, IARI, New Delhi on May 26, 2005.
- Seeds of these two varieties were also treated with 0.2 and 0.3 per cent ethyl methane sulphonate (EMS).
- Atleast 2000 seeds of each physical and chemical mutagen treatments in both varieties were sown at the Experimental Farm, HAREC, Kukumseri (Lahaul & Spiti) in May, 2005 to raise desirable size of M_1 generation.
- Seeds of individual M_1 plants were harvested during September- October, 2005. Seeds of only around 400 individual plants could be harvested in each of the treatment due to severe infection of *Fusarium* wilt disease under field conditions.
- For selecting desirable *Fusarium* wilt resistant mutants in the M_2 generations in each variety (physical as well as chemical mutated), the plant- to- row progeny of individual harvested plants were raised by sowing in sick plots under poly house conditions in November, 2005.
- The plots were also inoculated with *in vitro* multiplied inoculum of virulent isolates of *Fusarium oxysporum* f. sp. *pisi* prior to sowing to avoid any escape. The inoculum will also be added during the active crop growth stage to avoid any escape.
- In addition, atleast 2000 seeds each treated with gamma irradiation (15 and 20 KR) and EMS (0.2 and 0.3 %) in both varieties were also sown to increase the size of M_1 generation under field conditions at Vegetable Farm,

Department of Vegetable Science & Floriculture, CSKHPKV, Palampur on 24.11.2005.

- The seeds irradiated with gamma rays at Nuclear Research Laboratory, Indian Agricultural Research Institute, New Delhi did not germinate. Therefore, the seeds had to be irradiated again and were sown in January, 2006.

In vitro:

METHODOLOGY

Plant material:

Two varieties of pea *viz.*, Arkel and Azad P1 were used.

Explants:

Seeds of two varieties of pea *viz.*, Arkel and Azad P1 were used as explants for regeneration of *in vitro* plants. Epicotyl, nodal segments and leaf segments of *in vitro* grown 10-days-old seedlings of pea were used as explants for callus induction.

Culture Medium:

Murashige and Skoog (1962) medium (MS) was used as basal medium in all the experiments. The pH of the media was adjusted to 5.6-5.8 with either 0.1N HCl or 0.1N NaOH prior to autoclaving. The media were steam sterilized for 20 min at 1.1 kg/cm^2 pressure and 121^{0}C temperature. Composition of MS medium is as follows:

Composition of Murashige and Skoog (1962) medium

Component	Concentration (mg/l)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_20$	370

KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4.4H_20$	22.3
$ZnSO4.7H_20$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuS0_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
$FeSO_4.7H_20$	27.8
$Na_2EDTA.2H_20$	37.3
Meso-inositol	100
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Nicotinic acid	0.5
Glycine	2.0

Surface Sterilization:

Mature and healthy seeds were surface sterilized by immersing in 70 % ethanol for 2 min, and in 0.1 % $HgCl_2$ for 8 min. The sterilization process was followed by rinsing three times with autoclaved distilled water.

Inoculation:

Five surface sterilized seeds of each variety were inoculated in each 250 ml flask containing half strength MS (Murashige and Skoog, 1962) basal medium solidified with 0.8% agar.

Callus Induction:

Epicotyls, nodal segments and leaf segments of *in vitro* grown 10-days-old seedlings of pea were inoculated on callus induction medium. For callusing two media used are as follow:

i) MS + 2 mg/l NAA + 0.5 mg/l BAP + 3% sucrose + 0.8% agar (**C1**)

ii) MS + 1 mg/l NAA + 0.2 mg/l Kinetin + 5 mg/l 2, 4-D + 2 g/l Casein hydrolysate + 3 % sucrose + 0.8% agar (**C2**)

Shoot Regeneration:

After 30-40 days calli were transferred to the following shoot regeneration media:

i) MS + 1.86 mg/l NAA + 2.25 mg/l BAP + 3% sucrose +0.8% agar (**S1**)

ii) MS + 0.02 mg/l NAA + 2.25 mg/l BAP + 3% sucrose + 0.8% agar (**S2**)

Root Regeneration:

After 30-40 days regenerated shoots were transferred to root induction medium. Seven media tried for rooting are as follow:

i) MS + 2 mg/l NAA +3% sucrose + 0.8% agar (**R1**)

ii) MS + 2 mg/l IAA + 3% sucrose + 0.8% agar (**R2**)

iii) MS + 2 mg/l IBA +3% sucrose + 0.8% agar (**R3**)

iv) ½ MS + 0.25 mg/l IBA + 3% sucrose + 0.8% agar (**R4**)

v) ½ MS + 2 mg/l IAA + 3% sucrose + 0.8% agar (**R5**)

vi) ¼ MS + 0.25 mg/l IBA +3% sucrose + 0.8% agar (**R6**)

vii) ½ MS + 2 mg/l IAA + 3% sucrose + 0.8% agar (**R7**)

All these cultures were incubated at 25±1^{0}C temperature with 16/8 hrs light/dark cycle.

Suspension Culture:

Some calli (both friable and compact) of both the varieties were also transferred to Veliky and Martin, 1970 67-V cell suspension medium supplemented with 2% sucrose and PGRs *viz.*, 1.5 mg/l 2,4-D, 0.1 mg/l NAA,1.0 mg/l IAA and 0.25 mg/l

kinetin for cell proliferation. The pH of the media was adjusted to 4.5 with either 0.1N HCl or 0.1N NaOH prior to autoclaving. The medium was steam sterilized for 20 min at 1.1 kg/cm^2 pressure and 121^{0}C temperature. These suspension cultures were kept in the shaker at 120 rpm at 25± 2^{0}C. The composition of medium is as follows:

Composition of Veliky and Martin (1970) 67-V medium

Component	Concentration (mg/l)
KNO_3	800
$CaCl_2.2H_2O$	200
$MgSO_4.7H_20$	250
KCl	200
$NaH_2PO_4.H_2O$	150
$Na_2HPO_4.2H_2O$	20
$(NH_4)_2SO_4$	100
H_3BO_3	5
$MnSO_4.4H_20$	5.27
$ZnSO4.7H_20$	1.5
KI	0.05
$Na_2MoO_4.2H_2O$	0.25
$CuS0_4.5H_2O$	0.25
$CoCl_2.6H_20$	0.25
$FeSO_4.7H_20$	13.9
$Na_2EDTA.2H_20$	18.6
Meso-inositol	0.1
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Nicotinic acid	1.25
Ca pantothenate	1

RESULTS:

Standardization of protocols for

(a) Callus Induction:

Epicotyl, stem and leaf explants were inoculated on two types of callus induction media under aseptic conditions (Table 1). Cultured flasks were incubated at 25 ± 1^{o}C with 16 hrs light/dark cycle. Callus induction started in both the combinations of culture media i.e. C1 and C2 for all the explants (Plate 1 a). Green and compact

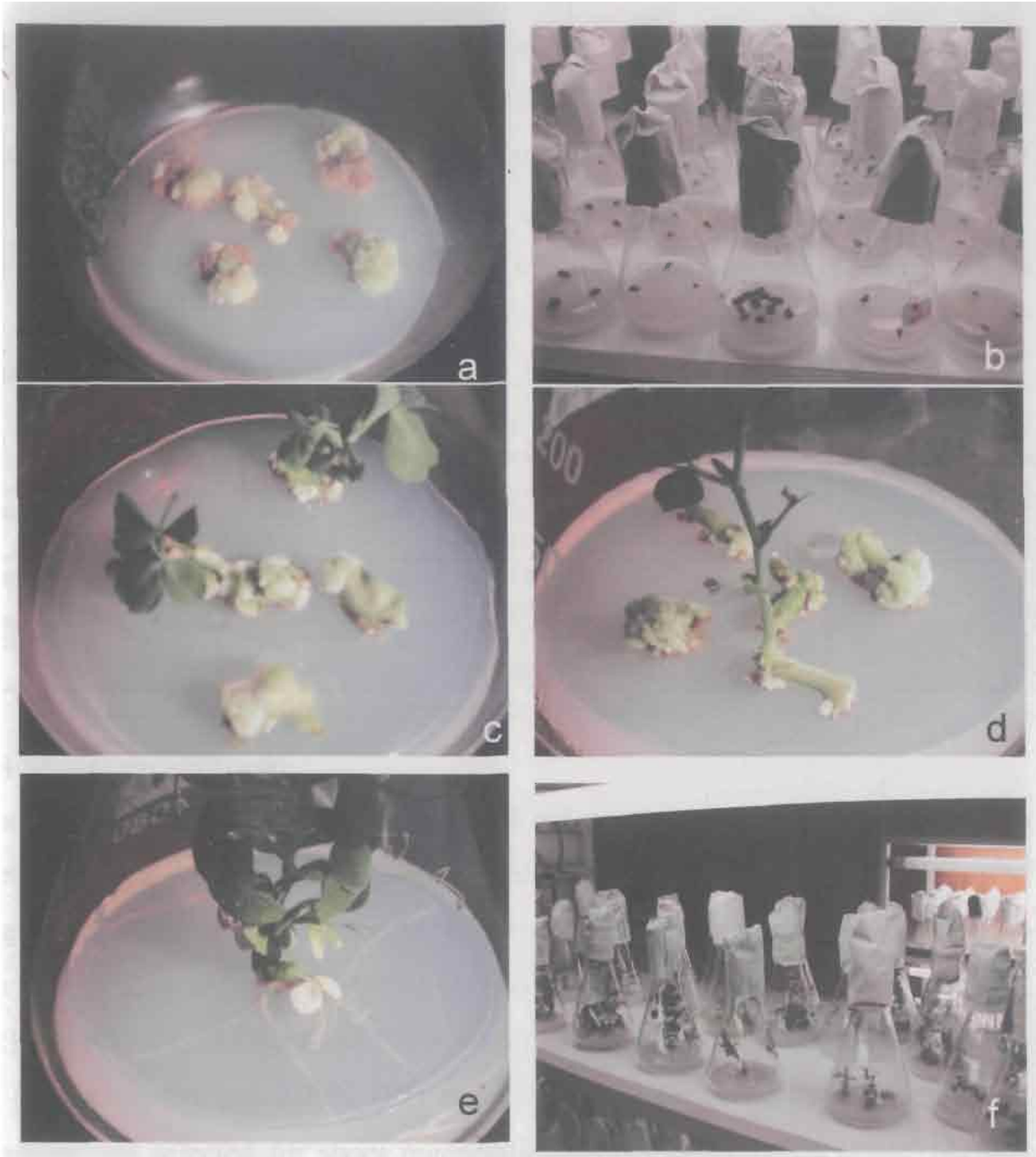

Plate 1

a: Callus induction in stem segments of pea.

b: Calli maintained on medium.

C & d: Shoot induction in callus.

e: Root induction in shoots.

f: Regenerated plantlets of pea variety Arkel.

calli were obtained on medium 'C1', while light green yellowish friable calli were obtained with medium 'C2' for both the varieties. Calli of two varieties of pea *viz.*, Arkel and Azad P1 from two media (C1 and C2) were subcultured on C1 medium after every 7-8 weeks. Around one thousand calli are maintained on the medium which will be screened for *Fusarium* toxin (Plate 1b).

Table1: Callusing response in different explants of Arkel and Azad P1 in two callus induction media

Explant	Callus induction medium (C1)				Callus induction medium (C2)			
	Arkel		Azad P1		Arkel		Azad P1	
	Explants inoculated (no.)	Calli formed (no.)	Explants inoculated (no.)	Calli formed (no.)	Explants inoculated (no.)	Calli formed (no.)	Explants inoculated (no.)	Calli formed (no.)
Stem	86	86 (100%)*	96	96 (100%)	52	52 (100%)	35	35 (100%)
Leaf	76	76 (100%)	68	68 (100%)	28	28 (100%)	26	26 (100%)
Epicotyl	6	6 (100%)	4	4 (100%)	5	5 (100%)	-	-

*The values in parentheses are percentage response.

(b) Shoot regeneration:

Shoot regeneration medium was standardized using 30-40 days old calli, which were transferred to shoot regeneration media *viz.*, S1 and S2. S2 medium showed higher rate of shoot regeneration for both the varieties as compared to S1(Plate 1c) (in case of stem segment 28.16% (S2) and 17.30 % (S1) for Arkel and 21.73 %(S2) and 8.57 %(S1) for Azad P1, in case of leaf explants 26.78% (S2) and 10.71 % (S1) for Arkel and 2.70% (S2) and 0.0% (S1) for Azad P1). Therefore, S2 medium was selected for shoot regeneration. Shoot proliferation (Plate 1d) was more in stem segments (17.30 % (S1) and 28.16% (S2) for Arkel and 8.57% and 21.73% for Azad P1) as compared to leaf segments (10.71% (S1) and 26.78% (S2) for Arkel and 0.0% (S1)and 2.70% (S2) for Azad P1) (Table 2). In epicotyl explants no shoot regeneration was observed.

Table 2: Shoot induction in calli originating from different explants of Arkel and Azad P1

Explant	Shoot regeneration medium (S1)				Shoot regeneration medium (S2)			
	Arkel		Azad P1		Arkel		Azad P1	
	Calli inoculated (no.)	Shoots regenerated (no.)	Calli inoculated (no.)	Shoots regenerated (no.)	Calli inoculated (no.)	Shoots regenerated (no.)	Calli inoculated (no.)	Shoots regenerated (no.)
Stem	52	9 (17.30%)*	35	3 (8.57%)	71	20 (28.16%)	46	10 (21.73%)
Leaf	28	3 (10.71%)	26	0 (0.0%)	56	15 (26.78%)	37	1 (2.70%)
Epicotyl	6	0 (0.0%)	4	0 (0.0%)	5	0 (0.0%)	-	-

* The values in parentheses are percentage response.

(c) Root regeneration:

Shoots were transferred to seven rooting media for standardizing the root regeneration medium. Of all the rooting media tested only one medium R4 proved successful for root regeneration (Table 3). Root production generally occurred on stem segment regenerating calli (Plate 1e). These results suggested that stem segments were better as explant for higher rate of root regeneration for both the varieties. Among all the regeneration media tested for the shoot and root regeneration S2 and R4 media, respectively will be used in future for shoot and root regeneration.

Table 3: Rooting response in shoots of AzadP1 and Arkel

Rooting medium	Azad P1				Arkel			
	Shoots inoculated (no.)		Roots formed (no.)		Shoots inoculated (no.)		Roots formed (no.)	
	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
R1	-	10	-	NR	-	-	-	-
R2	-	20	-	NR	-	20	-	NR
R3	15	20	NR	NR	-	20	-	NR
R4	-	-	-	-	25	-	20	-
R5	-	-	-	-	15	-	NR	-
R6	-	-	-	-	15	20	NR	NR
R7	5	-	NR	-	15	15	NR	NR

NR = No Response

(d) Suspension Culture:

Results of suspension culture are awaited.

Appointments: Both the Senior Research Fellows have joined the project in May, 2005 immediately after release of funds by the ICAR.

Name	Date of joining	Qualification
1. Dorin Gupta (For *in vitro* work)	06-05-2005 (resigned on 16.10.2005)	Ph.D. in Plant Breeding & Genetics
2. Sanjeev Pathak (For *in vivo* work)	13-05-2005	Ph.D. in Vegetable Science
3. Ms Vandana Patial (for *in vitro* work)	28.12.2005	M.Sc in Agric. Bio-technology)

Purchases and work completed by utilizing Non- recurring contingency:

- The Renovation work of exiting lab facilities is in progress. The work has been carried out by the Estate Cell of the University.
- Naturally ventilated polyhouse of size 15m X 7m with sprinkler irrigation facility has been installed in the Vegetable Farm, Department of Vegetable Science & Floriculture, CSKHPKV, Palampur.
- -20^0C Deep Freezer of "Make REMI" of volume 650 liters has been purchased.
- The case for repair of existing green house facilities has been sent for approval to the Central Purchase Committee of the University and the work will be completed as and when financial approval is received.

Facilities required:

- The **institutional charges are allocated less than the actual estimates** since these are also calculated on the salaries of SRF which are otherwise not included in the total budget allocation. So, the amount less computed is **Rs. 61,860/-** only which may **kindly be get released at the earliest.**

- **"Digital Camera with accessories"**- This will help to get good quality photographs of different mutants to be generated in the coming years of the project study and will improve the quality of the research work.
- **"Autoclave"-** This will help to sterilize the culture medium for *in vitro* disease multiplication to be used for artificial inoculation of soil to avoid any disease escape.
- **"Computer and its accessories alongwith inter- net facilities"-** To gather recent up to date information and literature pertaining to research project, data analysis, timely report preparation etc.

Signatures:

2.2.2006

Principal Investigator

2.2.2006

Head
Department of Vegetable Science & Floriculture,
CSKHPKV, Palampur

3-02-06

Director of Research,
CSKHPKV, Palampur