

Original Research Article

Host-Parasite Relationships in Tissue Culture of Safflower and *Fusarium oxysporum* f. sp. *carthami*

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ABSTRACT

Among the different diseases affecting safflower, wilt disease caused by *Fusarium oxysporum* f. sp. *carthami* observed in severe form, this disease spread widely and causes yield losses up to 80%. Microscopy was developing reliable *in vitro* assays may provide a basis for the use of interactions in dual cultures *in vitro* to evaluate the pathogenicity of fungi and susceptibility of host plant genotype. The pathogenicity of fungi and susceptibility of host plant genotypes along with the degree of threat posed by fungi was evaluated. This may also enable the selection on embryonic level that is more resistant to the pathogen, which is particularly important for fungi known for their ability of epiphytic occurrence. The margin of the leaf lamina were cultured in the MS medium supplemented with different concentration 0.5 to 2.5 mg/L of 2,4-D there was formation of abundantly growing callus, in concentration of 2,4-D (2.0 mg/L) callus on entire surface of leaf. After 3-4 weeks few green nodules were observed on the surface of white transparent nodulated callus. Changes due to *Fusarium oxysporum* f. sp. *carthami* fungus infection to callus give reliable results which can be used for selection of disease resistant genotype. The present investigation was conducted for standardization of *in vitro* callus induction protocol conducted for the variety GMU and to study effect of *Fusarium carthami* on callus of safflower at cellular level by microscopic study.

Keywords

Safflower,
Fusarium
carthami,
Host-parasite,
Pathogenicity,
2, 4-D, GMU

Introduction

Safflower (*Carthamus tinctorius* L.), an oilseed crop is a member of the family Compositae or Asteraceae. *Carthamus* is Latinized synonym of the Arabic word *quartum* or *gurtum*, which refers to the color of the dye extracted from safflower flowers. The English name *safflower* probably evolved from various written forms of *usfar*, *affore*, *asfiore* and *saffiore* to *safflower*. Safflower has been grown in India since time immemorial. It is mentioned as *kusumba* in ancient scriptures. Presently, in India it is most commonly known as 'kardai' in Marathi and 'kusum' in Hindi. *Carthamus*

has 25 species, of which only *C. tinctorius* is the cultivated type, having 2n=24 chromosomes. Safflower has high adaptability to low moisture conditions. Therefore, its production all over the world is mainly confined to areas with scanty rainfall. The crop has tremendous potential to be grown under varied conditions and to be exploited for various purposes. Safflower, a multipurpose crop has been grown for centuries in India for the orange red dye, carthamin extracted from its brilliantly colored flowers and for its quality oil. Traditionally, the oil has mainly been

sold in the health food market because of its unsaturated oil content, linoleic acid (67-83%) and oleic acid levels (74-80%) (Nimbkar, 2002). Safflower flowers are known to have many medicinal properties for curing several chronic diseases and are widely used in Chinese herbal preparations (Klisiewicz, 1962). The tender leaves, shoots, and thinning of safflower are used as pot herb and salad. They are rich in vitamin A, iron, phosphorus and calcium. Bundles of young plants are commonly sold as a green vegetable in markets in India and some neighboring countries (Nelson, 1981). Safflower can be grazed or stored as hay or silage. Safflower forage is palatable and its feed value and yields are similar to or better than those for oats or alfalfa. Thus, each part of safflower has a value attached to it. The area under safflower around the world is limited largely due to the lack of information on its crop management and product development from it (Nikam *et al.*, 1999).

Distribution and production

Traditionally, safflower has been grown for centuries from China to the Mediterranean region and all along the Nile valley up to Ethiopia (Oyen, *et al.*, 2007). India is ranked number one in global safflower production and acreage. India accounts for 3,00,000 ha (41%) of area under safflower cultivation globally. However, owing to one of the poorest productivity, Indian safflower production at 1.89 lakh tonnes is only 29% of global production followed by the US (17%), Argentina (13%) and Kazakhstan (12%). Top five countries contributed more than 76% of world's production in 2009 with about 80% of global safflower acreage. Safflower occupies the seventh place in an area dedicated to oilseeds in India. Nearly, 99% of the area under the crop is located in the southern plateau. However, area under

safflower cultivation has declined by 64% since 1991 and is continuously declining over the last 10 years. Production has also declined by 41 per cent during the same period (Booth, 1971).

In India key safflower producing states are Maharashtra and Karnataka contributing 55% and 31% respectively. Maharashtra, Karnataka along with Gujarat and Andhra Pradesh accounts for 94 per cent of Indian area under safflower cultivation and about 99 per cent of country's safflower production. Gujarat has highest productivity (1,000 kg/ha) of safflower in India followed by Karnataka (795 kg/ha), Maharashtra (565 kg/ha) and Andhra Pradesh (462 kg/ha) among major safflower producing states (Booth, 1971).

Reasons for decrease in safflower production

Many bacterial, fungal and viral pathogens infect the safflower crop causing diseases *viz.*, bacterial leaf spot and stem blight caused by *Pseudomonas syringae* van Hall, *Alternaria* blight by *Alternaria carthami*, leaf spot by *Cercospora carthami*, wilt by *Fusarium oxysporum*, rust by *Puccinia carthami*, powdery mildew by *Erysiphe cichoracearum*, mosaic by Cucumber mosaic virus (CMV) which affect the crop production. Among all these *Fusarium* wilt is observed as one of the most common and destructive disease in safflower. Being soil borne in nature, the fungus survives in the soil as chlamydo spores in diseased plant debris without losing viability (Business Line, 2011). *Fusarium oxysporum* f. sp. *carthami* invades the tap root of safflower directly or through its root hairs by mechanical means. The disease becomes severe when the plant reaches about 15 cm in height. The increase in disease incidence results from a continuous cultivation of

safflower i.e. mono cropping and re-use of infected seeds harvested from wilt infected plants, which contributes to increasing the primary source of the inoculum (Jarvis, 1978). Disease severity seems to be directly related to the use of susceptible varieties and a large population of *Fusarium oxysporum* f. sp. *carthami* in the soil (Anonymous, 2002). The variation in temperature and soil moisture exerts an influence on the fluctuation of the disease expression and severity. Moreover, crop growth stages right from seedling to post flowering stage play an important role in the incidence and expression of wilt in safflower.

Fusarium, a mitosporic *hypocreales* fungal genus, various species of which are important parasitic pathogens of plants, is a diverse genus consisting of an array of species responsible for damping-off, root rots, and vascular wilts on a multitude of economically important plant species (Murashige and Skoog, 1962). The confusion regarding the taxonomy of *Fusarium* is perhaps best illustrated by the diversity of proposed taxonomic systems (Nelson, 1991).

Fusarium oxysporum Schlecht is an extremely common soil fungus that occurs in the rhizosphere of many plant species. Most *F. oxysporum* strains in soil live saprophytically on organic substrates. However, some soilborne *F. oxysporum* strains can cause plant diseases, especially root rots and wilt disease. Strains of some *Fusarium* species are effective as biocontrol agents. *F. oxysporum* is a very complex group, divided into *formae speciales* and physiological races depending on the pathogenicity toward particular plant species or cultivars (Armstrong, 1981).

Wilt of safflower is mainly caused by *Fusarium oxysporum* f. sp. *carthami*. This

pathogen penetrates the roots mainly through wounds and proceeds into and throughout the vascular system, leading to functional collapse, systemic wilting and often death of the infected plant. Infected plants may either totally wilt and die, or persist in a weakened state, producing a reduced number of inferior fruits (Griffin *et al.*, 1968).

Fusarium species produces chlamydo spores, macro conidia and micro conidia. All stages are present in infected tissues or soil (Nelson, 1991). Details of the colonization process of *Fusarium* species within and outside the vascular system has been studied (Nimbkar, 2008).

The mycelial development in the vicinity of plant roots has been investigated (Olivain, 1997), but little is known about the germination of *Fusarium* species propagules, a key step in plant pathogen interactions (Murashige and Skoog, 1962).

In the view of above constraint, the present study being proposed to take initiatives for following objective.

To study the callus induction of safflower by leaf explants on different 2, 4-D concentration.

To study the host-parasite relationships in tissue cultures of safflower and *Fusarium oxysporum* f. sp. *carthami*.

Materials and Methods

Experimental material safflower genotype and *Fusarium oxysporum* f. sp. *carthami* culture collected from A.I.C.R.P. on Safflower, Parbhani. Leaf explants was taken from 30 days old seedlings, germinated on the moist soil and sand (1:1) germination in the pots.

Source of explants

For initial establishment of callus culture, Leaves from the 3 week old in greenhouse grown seedlings were used as the source of explants.

Micro propagation of Safflower

Cleaning and disinfection

The glasswares required for this experiment were washed with labolin detergent followed by disinfection in solution containing potassium dichromate and concentrated H₂SO₄. The disinfection solution was prepared by dissolving 60g of potassium dichromate in 35 ml of distilled water, heated for 2-3 minutes and final volume was made to one liter by adding conc. H₂SO₄. On subsequent day disinfected glasswares were thoroughly washed in tap water, rinsed with distilled water and immersed into diluted solution for overnight.

Finally the cleaned, disinfected glasswares were allowed to dry on draining racks, until their sterilization.

Sterilization of glasswares

The glasswares viz. culture tubes, bottles, Petri dishes, pippets, beakers, measuring cylinder, conical flask etc. for sterilization of culture tubes and bottles they were closed with non-absorbent cotton and caps.

Petri dishes, pipettes, beakers, measuring cylinder, conical flask etc. were sterilized by wrapping by wrapped in an aluminium foil and kept in wire mesh basket prior to autoclaving. The wire mesh basket containing glasswares were autoclaved at 15lbs. at 121⁰C for a 30 min followed by drying in hot air oven at 80-100⁰C for 1hr. for removal of excess moisture. Forceps and

scalpel like instruments were sterilized by flame sterilization technique. The culture showing unwanted microbial growth (contamination) was discarded after autoclaving in order to destroy the source of contaminants.

Preparation of stock solution

The stock solutions of major and minor elements (8X) and vitamins (50X) were prepared in sterile distilled water. The quality of major elements, minor elements and vitamins were measured as per description given in Table 2, dissolved thoroughly in sterile H₂O with the help of magnetic stirrer and stored at 4⁰C. These stock solutions were routinely used in preparation of various media composition required in micro propagation of safflower.

Also the stock solution of growth regulators like 2, 4-D and NAA were prepared by dissolving their required quantity in a few drops of 1N NaOH and final volumes made up with sterile water, filter sterilized and stored in refrigerator at 4⁰C.

The explants were cultured on MS medium with various combinations of growth regulators as shown below.

Procedure for preparation of MS culture media

One litre of MS basal media was prepared by taking 50 ml of stock of major nutrient (MA 8X) was transfer to clean and sterile beaker.

To the same beaker 5 ml of stock of minor (MB 50X) was added and stirred well with help of magnetic stirrer.

The major source of carbon (sucrose) was added at a conc. of 3% and dissolved to the

medium while continuous stirring, the volume of solution was made up to 900ml with doubled distilled water.

The pH of the medium was adjusted to 5.8 by using 0.1N HCl and 0.1 N NaOH before sterilization.

The final volume of solution was made up to one litre by using doubled distilled water.

The solidifying agent, Agar-agar was added to the medium at 0.8% concentration and mixed thoroughly to the medium by gently heating of the media.

The required quantity of media approximately 20-30 ml per bottle was dispensed and labeled properly.

The bottles containing culture media were kept in wire mesh basket, wrapped with aluminum foil and autoclaved at 121⁰C at 15 lbs PSI pressure for 20 min.

After sterilization the media was allowed to cool and then required volume of growth regulators (2, 4-D, NAA) from stock solution were added separately in each bottle in laminar airflow cabinet under aseptic condition.

The bottles were sealed with parafilm and stored at 25⁰C until use.

Selection and sterilization of explants

The mother plant selection on the basis of their genetic purity, high yield performing, free from viral disease and pest and vigorous growing habit.

Collect the leaves from a selection elite plant.

Wash the leaves under tap water.

Surface sterilized with 0.7% HgCl₂ for 2-3 min.

Wash thoroughly 2-3 times with single distilled water.

Transfer the in flask containing 70% ethanol for 1 min. and immediately wash with sterile water.

To avoid bacterial contamination, use of Cefataxime (0.1%) in the initiation medium is in vogue in some laboratories.

If virus elimination is required, using dissecting microscope and scalpel.

Culture room

The explants were incubated in a culture room where the temperature was maintained at 24±1 °C, humidity at 70 % and under a photoperiod of 16 h light and 8 h dark.

Initiation of explants

Take leaves in sterile Petri plate.

Cut the leaf with the help of forceps and scalpel carefully in to small pieces in a size of explants and inoculate.

The explants on callus establishment medium in culture bottle and incubate at a 25+2⁰C in a dark for a week.

Transfer the culture in light (100 lux) and incubate given a 16 hrs photoperiod at a 25⁰C.

Transfer these culture 7-8 days of incubation to a fresh medium in the laminar airflow chamber.

Observe the culture regularly and discard the contaminated culture.

Collection and maintenance of *Fusarium* isolates

Fusarium oxysporum f. sp. *carthami* culture is obtained from A.I.C.R.P. on safflower V.N.M.A.U., Parbhani. The isolates of the pathogen were identified based on characters and spores morphology (Booth, 1971). The isolate was maintained on PDA medium at 4°C and subcultured after every 3 months.

Establishment of dual cultures (Fungus – Callus)

Dual cultures were established in sterile culture bottle with solidified callus-proliferation medium.

Fungal inoculum of 0.5 x 0.5 cm was placed in the center of the sterile culture bottle while the host-plant callus with a diameter of 1.0 - 1.5 cm and weighing 500 mg should be put 5.0 mm at the centre of the bottle (Flor, 1955).

Microtomy of *Fusarium* infected callus

Fusarium infected callus and callus without infection were fixed into the melted paraffin wax. Wait for one day for solidification of wax. Finally thin sections of the callus fixed into paraffin block were made by microtome.

Cells were fixed and stained with Lactophenol and cotton blue

The Integrity of cellular structures such as the nucleus and plasma membrane and the presence of intracellular fungal structures such as haustoria or infection vesicles can be confirmed with following stains.

Cells were stained with Janus Green B, which is absorbed only by living cells.

Cells were stained with Aniline-blue and illuminated with fluorescent light, which stain callus.

After 20 h, callus of safflower inoculated with *Fusarium* examined by microscopy to verify the presence of fungal infection structures on the surface of the host cells.

Results and Discussion

The results of present study entitled “Host-parasite relationships in tissue culture of safflower and *Fusarium oxysporum* f. sp. *carthami*” were described in this chapter. In this investigation effect of fungal infection on callus of the safflower was done. The results were presented under following headings.

In vitro callus induction is an important step in the success of any crop improvement programme through biotechnology as well as Microscopy is a well-defined, reproducible and highly efficient method for study the host-parasite relationships in tissue culture of crop plants.

The safflower cv. ‘GMU-5080’ was screened for callus induction and microscopic analysis of *Fusarium oxysporum* f. sp. *carthami* infection. The following steps were performed i.e. germination of safflower, explants isolation and regeneration, dual culture (callus+ fungus), microscopy (Figs 1–5).

Germination of safflower

The variety GMU-5080 and *Fusarium oxysporum* f. sp. *carthami* culture was used for *in vitro* callus induction and infection studies were collected from A.I.C.R.P. on Safflower, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani. Seeds were soaked in water for about 48 hrs; helps to

break dormancy. Soaked seeds were germinated on the moist soil and sand (1:1) germination in the pots.

Callus induction by leaf explant

In this experiment leaf was used as an explant measuring 0.5 to 1 cm was aseptically transfer into culture medium for callus induction with 8 different media combination, consisting of MS basal formulation as below.

All the eight different media combinations were supplemented with vitamins, thiamine and meso-inositol. In this experiment, leaf explants were inserted into the medium in such a way that the lower portion of leaf was vertically inserted into the medium and upper portion remains above the surface of medium. After 30 days of culture, leaf explants increase slightly in volume and subsequently the compact callus was formed at the cut edges of the each explant. The callus was observed to be more in full MS + 2, 4-D (2ppm) as compared to other growth hormone combinations consisting of 2, 4-D (Table 4). While explants in remaining media combinations did not give better response to callus induction. From Table 3, it was clear that *cv* GMU-5080 responded more to callus induction on MS + 2, 4-D (ppm).

Maintenance of fungus culture

Fungal culture was maintained on PDA medium at 4°C and subculture after every 3 months.

Infection of fungal culture to safflower callus

Fungal infection to callus was done in sterile culture bottle with solidified callus proliferation medium. Fungal inoculum of

0.5 x 0.5 cm should be placed in the center of the sterile culture bottle while the host-plant callus with a diameter of 1.0 - 1.5 cm and weighing 500 mg should be put 5.0 mm from the edge of the bottle.

Effect of *Fusarium oxysporum* f. sp. *carthami* on callus at cellular level

Fusarium fungus infection to the callus studied under microscopy gives characteristic changes at cellular level.

Thick sections revealed that the fungus was concentrated in the outermost cell layers of the callus. No haustoria were seen inside the callus cells.

Infection structures formed on the surfaces of cells derived from resistant safflower callus.

No intracellular fungal structures or other signs of penetration were evident. There was no indication that cells inoculated with incompatible races of *Fusarium oxysporum* f. sp. *carthami* underwent rapid death within 20 hours of inoculation.

Chlamydospores insisted on resistant hosts germinated and produced structures.

Safflower (*Carthamus tinctorius* L.), an oilseed crop is a member of the family Asteraceae.

Safflower wilt is a major disease caused by the genus *Fusarium oxysporum* f. sp. *carthami* culture is a widely distributed phytopathogen and brings about great economic loss of crops.

In vitro callus induction media was standardized. Different media combinations with various hormonal concentrations were used. It can be concluded from Table 4 that

the leaf explants of safflower in MS + 2, 4-D (2 ppm) show maximum callus induction.

The early stages of infection of safflower callus by *Fusarium* were observed by microscopy. Microtomy and microscopic analysis of inoculated callus revealed the presence of the fungus only on outer surface of the callus. Thick sections revealed that the fungus was concentrated in the

outermost cell layers of the callus. No haustoria were seen inside the callus cells.

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Table.1 Variety of safflower used in programme

Name of Variety	:	Resistant/Susceptible
GMU-5080	:	Resistant

Table.2 Preparation of stock solution of major and minor nutrients (8X) and vitamin nutrients (50X) as per composition given by MS Medium

Stock I:Major Element			50ml
		Qty (mg/l)	
Ammonium nitrate	NH ₄ NO ₃	1650.00	
Potassium nitrate	KNO ₃	1900.00	
Calcium chloride	CaCl ₂ . 2H ₂ O	440.00	
Magnesium sulphate	MgSO ₄ . 7H ₂ O	370.00	
Potassium dihydrogen orthophosphate	KH ₂ PO ₄	170.00	

Stock II :Minor Element			5ml
Potassium iodide	KI	0.83	
Boric acid	H ₃ BO ₄	6.20	
Manganese sulphate	MgSo4	22.30	
Zinc sulphate	ZnSO ₄ 7H ₂ O	8.60	
Sodium molybdate	Na ₂ MoO ₄ 6H ₂ O	0.25	
Copper sulphate	CuSO ₄ 5H ₂ O	0.025	
Cobalt chloride	CoCl ₂ 6H ₂ O	0.25	

Stock III			5ml
Sodium EDTA	Na ₂ EDTA	37.30	
Ferrous sulphate	FeSO ₄ 7H ₂ O	27.85	

Stock IV: Vitamins and other			5ml
Inositol	C ₆ H ₁₂ O ₆	5000	
Glycine	C ₂ H ₅ NO ₂	100	
Thiamine HCl	C ₁₂ H ₁₇ CIN ₄ OS HCl	0.10	
Nicotinic acid		25	
Pyridoxine HCl		25	

Note: Dissolve ferrous sulphate and Na₂EDTA separately. Dissolve Na₂EDTA into warm sterile water and then add ferrous sulphate to the same solution and dissolved under continuous stirring.

Figure.1 Germination of safflower



Figure.2 Leaf explants on MS media

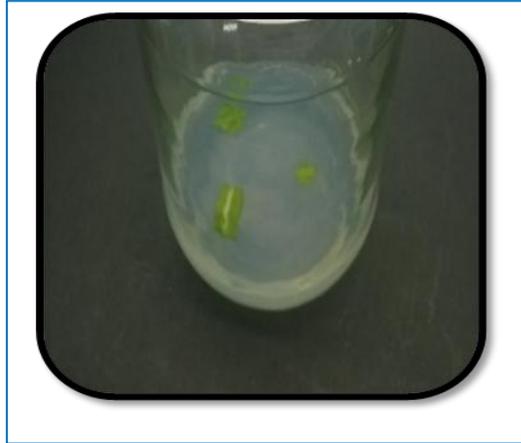


Figure.3 Callus induction (MS+2,4-D 2 ppm)



Figure.4 Dual cultures *in vitro* (callus- fungus): Safflower-Fusarium

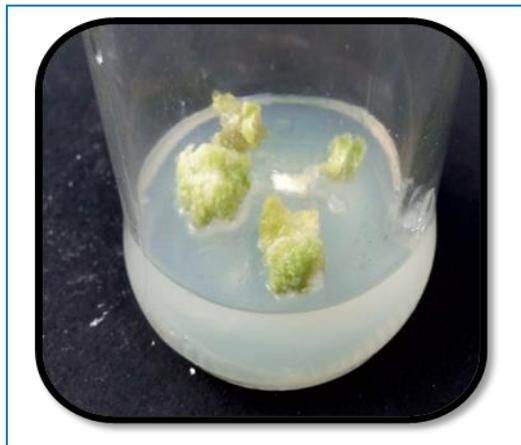


Figure.5 Microscopic analysis of callus infection

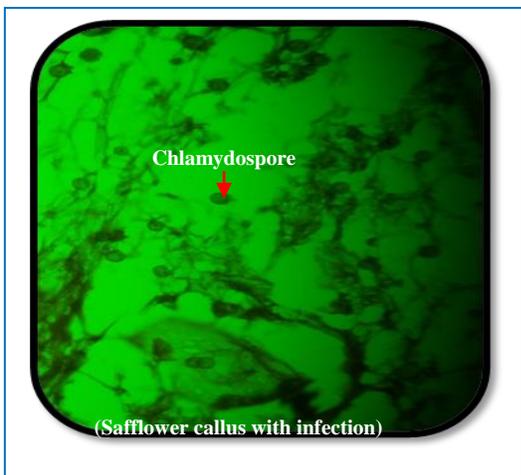


Table.3 Different growth regulator combinations for callus induction with standard MS medium

Combination no.	2,4-D (ppm)	NAA (ppm)
1	0	0
2	0.25	0.50
3	0.50	0.50
4	0.75	0.50
5	1.00	0.50
6	1.50	0.50
7	2.00	0.50
8	2.50	0.50

Table.4 Effect of different concentrations of 2, 4-D and NAA on callus induction of safflower explants after four weeks (30 days) of inoculation

Treatment	2-4, D (PPM)	NAA (PPM)	Callus Weight (gram)	Morphology of Callus
Control	0	0	0	No callus formed
2	0.25	0.5	1.0	No callus formed
3	0.50	0.5	1.6	White green, Friable
4	0.75	0.5	1.7	White green, Friable
5	1.0	0.5	1.9	Light green, Compact
6	1.5	0.5	2.3	Light green, Compact
7	2.0	0.5	2.5	Light green, Compact
8	2.5	0.5	1.3	Light green, compact

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