Indian J. Plant Physiol., Vol. 11, No. 1, (N.S.) pp. 36-40 (Jan.-Mar., 2006)

PRODUCTION OF DIPHENOL AND MONOPHENOL COMPOUNDS FROM THE LEAF CALLUS OF *MORUS ALBA* AND STUDIES ON THEIR ANTIBACTERIAL PROPERTIES

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Received on 3 Oct., 2005, Revised on 17 March, 2006

SUMMARY

White mulberry (*Morus alba* L.) is an economically important tree species of family Moraceae and is cultivated in many parts of the world. Leaf discs of white mulberry were used as explants and inoculated on to Murashige and Skoog (MS) medium supplemented with various concentrations of 2,4-D, NAA, BAP and KiN. Antibacterial activity was evaluated from explants and leaf induced calluses against *Bacillus subtilis*. Calluses raised from different combinations showed remarkable antibacterial activity against *Bacillus subtilis* as compared to explants alone i.e. leaf tissue. Phenolic contents from explants and calluses were also estimated. Diphenols and monophenols were higher in callus cells as compared to explants suggesting their probable role as antimicrobial agents against *B. subtilis.* Further, antibacterial activity against *B. subtilis* was also tested with standard monophenol and diphenol.

***Key words*: Antibacterial activity, *Bacillus subtilis,* leaf callus, *Morus alba,* phenols**

INTRODUCTION

Plant based drugs are being increasingly preferred in medical science. The use of medicinal plants / plant parts to cure specific ailments has been in vogue from ancient times in our indigenous medicine. It is, however, unfortunate that these plants are harvested from the site randomly without replacing them back in the ecosystem. It is also important to know physiological condition of the plant which may help in harvesting optimum concentration of medicinally useful compounds. These problems can be solved with the help of plant tissue culture techniques, where mass propagation of plant or plant parts under aseptic conditions yields desired compounds. Further, both quality and quantity of such compounds can be regulated in culture conditions.

Polyphenolic compounds, widely found in the plant systems, are reported as inhibitory lipid peroxidation and exhibit various physiological activities including anti-inflammatory, antiallergic, anticarcinogenic and antimicrobial activities (Paupponen – Pimia *et al*. 2001). White mulberry (*Morus alba* L.) is a tree with many economically important properties, specially it is a foliage crop to feed silkworm. Medicinally, this plant is used as refrigerant in fevers and locally used as remedy for sore throat, dyspepsia and melancholia (Reed 1976). Since one of the applications is for sore throat, in the present study, leaves of *Morus alba* were studied to test antibacterial activities against *Bacillus subtilis*. Further, leaf tissues were cultured *in vitro* to induce callus on different hormonal concentrations. Callus induced on different media were also tested for the study of antibacterial activities. Monophenols, diphenols and polyphenols were also studied from leaf explants and from leaf induced callus to understand the probable role of these phenolics in antibacterial activities against *B. subtilis*.

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MATERIALS AND METHODS

Explant preparation and cultural conditions: Fully expanded leaves were selected as explants and washed under running tap water for 3 h, surface sterilized with 0.1% $HgCl_2$ for 15 min and washed several times with sterile distilled water. From each leaf, leaf discs (1 cm in diameter) were cut and inoculated on basal medium containing various concentrations of 2,4-D, NAA, BAP and KiN (Table 1). Murashige and Skoog (1962) medium containing 0.8% agar and 3% sucrose was used as basal medium to induce callus from leaf discs. All the components of each nutrient medium were mixed and its pH was adjusted to 5.8 prior to autoclaving at 121°C, 104 kPa for 20 min. Percent callus induction was calculated to determine the optimum medium for callus induction. Observations were recorded up to six weeks to determine the effect of various concentrations of auxins and cytokinins and percent responses per explant were calculated with mean and ± standard deviation.

Antibacterial activity: Antibacterial activity of explant tissue or callus cells against *B. subtilis* was determined following the method of Bhatt *et al.* (2003). Plant tissue or callus tissue (500 mg/L) were crushed and added to nutrient agar before autoclaving. After sterilization media was poured in petri dishes (20-25 ml / dish) under laminar flow hood. Bacterial suspension (100 µl) of 10^{-4} dilution of *B. subtilis* was inoculated with the help of sterile glass spreader. Plates were kept overnight at room temperature for incubation. After 24 h, colonies were counted in each plate to determine the effect of plant or callus extract.

Estimation of the phenolic compounds: Total phenolic content was determined as per the method of Swain and Hills (1959). The standard curve was prepared using different concentrations (10-100 µg) of chlorogenic acid. The concentration of monophenols was determined using the method of Emerson (1943). The standard curve was prepared using different concentrations of ferrulic acid (25-250 µg). *O*-dihydroxy phenolic content was determined using the method of Mahadevan (1986). The standard curve was prepared with different concentrations of pyrocatechol (10-100 µg). Phenolic content was expressed as µg phenol/ g leaf tissue or g callus. Each sample was estimated in triplicate and the mean values were calculated with ± standard deviation.

Antibacterial activity of standard monophenol and diphenol: Antibacterial activity of standard monophenol and diphenol was tested with dichlorophenol and pyrocatechol respectively. Dichlorophenol was taken in a concentration of 2.5, 5.0, 10.0, 15.0 µg/ml. Pyrocatechol was taken in a concentration of 0.05, 0.075, 0.1, 0.15 µg/ml. These concentrations were set according to the concentrations of callus tested. Dichlorophenol and pyrocatechol with these concentrations were mixed with nutrient agar medium and autoclaved. After sterilization media was poured in petri dishes (20-25 ml / dish) under laminar flow hood. Bacterial suspension (100 µl) of 10^{-4} dilution of *B. subtilis* was inoculated with the help of sterile glass spreader. Plates were kept overnight at room temperature for incubation. After 24 h, colonies were counted in each plate to determine the effect of standard monophenol and diphenol.

RESULTS AND DISCUSSION

The role of plant growth regulators in induction of callus from various explants is extensively studied. In this study, various combinations of plant growth regulators were tested in different combinations. Callus induction started after second week of inoculation and percent callus induction was maximum when 5.4 µM NAA and 4.4 µM BAP were supplemented in the nutrient medium, whereas poor callus induction was observed when leaf discs were cultured on media with 9 µM 2,4-D and 4.6 µM KiN concentrations. The role of NAA as an effective source of auxin for callus formation and /or regeneration of shoots has been reported earlier in crop plants such as mustard (George and Rao 1989), *Brassica* (Wong and Loh 1987) and *Capsicum* (Subhash and Christopher 1988). It is interesting to note that NAA with 2.2 µM BAP or 4.6 µM KiN induced callus up to 75 to 100% from the explants, respectively. For cent percent induction of callus from explants less concentration of BAP is required as compared to KiN.

Callus induced from plant parts shows stable characteristics under specific conditions after subculture

through many successive passages (Narayanswamy 1994). For the production of higher amount of biologically active compounds, maximization of callus production is necessary in setting up an effective experimental system.

Explants and calluses induced from different media were tested for antibacterial activities against *Bacillus subtilis* (Table 1). Earlier studies from our laboratory, Bhatt *et al*. (2003) have demonstrated that direct addition of active compound(s) or biologically active material in nutrient media, helps to react with bacteria faster then that of classical paper disc or agar ditch method. Therefore, in this study, callus cells or equal amount of explants tissue (500 mg / L) mixed with N-agar showed distinct results (Table 1). Amongst the nine different callus masses grown on different culture media were tested, few calluses showed very strong antimicrobial activities, while rest has few colonies (Table 1). Out of nine different combinations, callus induced from 2,4-D in combination with KiN or BAP showed maximum antimicrobial activity; NAA and BAP or KiN induced callus showed few colonies, as compared to loan formation in control plates (Table 1, Plate 1). On the other hand, media containing explants showed good number of colonies nearly that of control plates. This result suggests that callus cells might have produced relatively higher amount of biologically active compound(s) responsible for antimicrobial activity against *B. subtilis* then that of leaf tissue (Table 1). The antibacterial activity of standard monophenol, i.e. dichlorophenol showed that with increasing the concentrations, the colony numbers were reduced (Table 2). At 10 µg/ml dichlorophenol with decrease in number of colonies, the size of colonies was also reduced. At higher concentration of 15 µg/ml, there was no growth. While with diphenol, i.e. pyrocatechol, with increasing the concentrations the number of colonies were increased and no correlation was observed.

The antimicrobial activities of the naturally occurring phenolics from olives, tea and wine have been widely studied (Ruiz-Barba *et al*. 1990; Vivas *et al*. 1997, Chou *et al*. 1999). When monophenols, diphenols and polyphenols were estimated from the explants and callus cells (Table 1) it was observed that, callus cells have more diphenols and monophenols as compared to explants, however, the total phenolic contents remained higher in explants tissue. This suggests the potential role

Table 1. Anti *Bacillus subtilis* activity of phenolic compounds, extracted from different callus induced on various concentrations of plant growth regulators

Media	PGR Conc. (µM)				Total Phenols	Monophenols	Diphenols	Colony Count
No.	NAA	2,4-D	BAP	KiN	(mg . g Fwt^{-1})	(mg . g Fwt^{-1})	(µg . g Fwt^{-1})	
1	5.4	0	2.2	0	1.53±0.18	2.64±0.03	37	1
2	5.4	0	0	2.3	2.46±0.16	3.83±0.23	81±1.41	0
3	0	2.3	0	4.6	2.25	3.95±0.15	73±31.11	0
4	0	4.5	0	4.6	2.44±1.02	2.59±0.16	62.5±7.78	0
5	0	6.8	0	4.6	2.65±0.20	8.20±1.68	95±21.21	0
6	0	9	0	4.6	1.50±0.28	6.28±0.13	63.5±2.12	0
7	2.7	0.9	4.4	0	1.59±0.51	1.33	22	2
8	5.4	0	0	4.6	1.58±0.33	3.41±0.46	46.5±3.45	126
9	5.4	0	4.4	0	1.18±0.06	1.83±0.35	25.5±4.95	10
Leaf Tissue					2.92±0.45	0.89±0.15	16.2±1.98	Non Countable

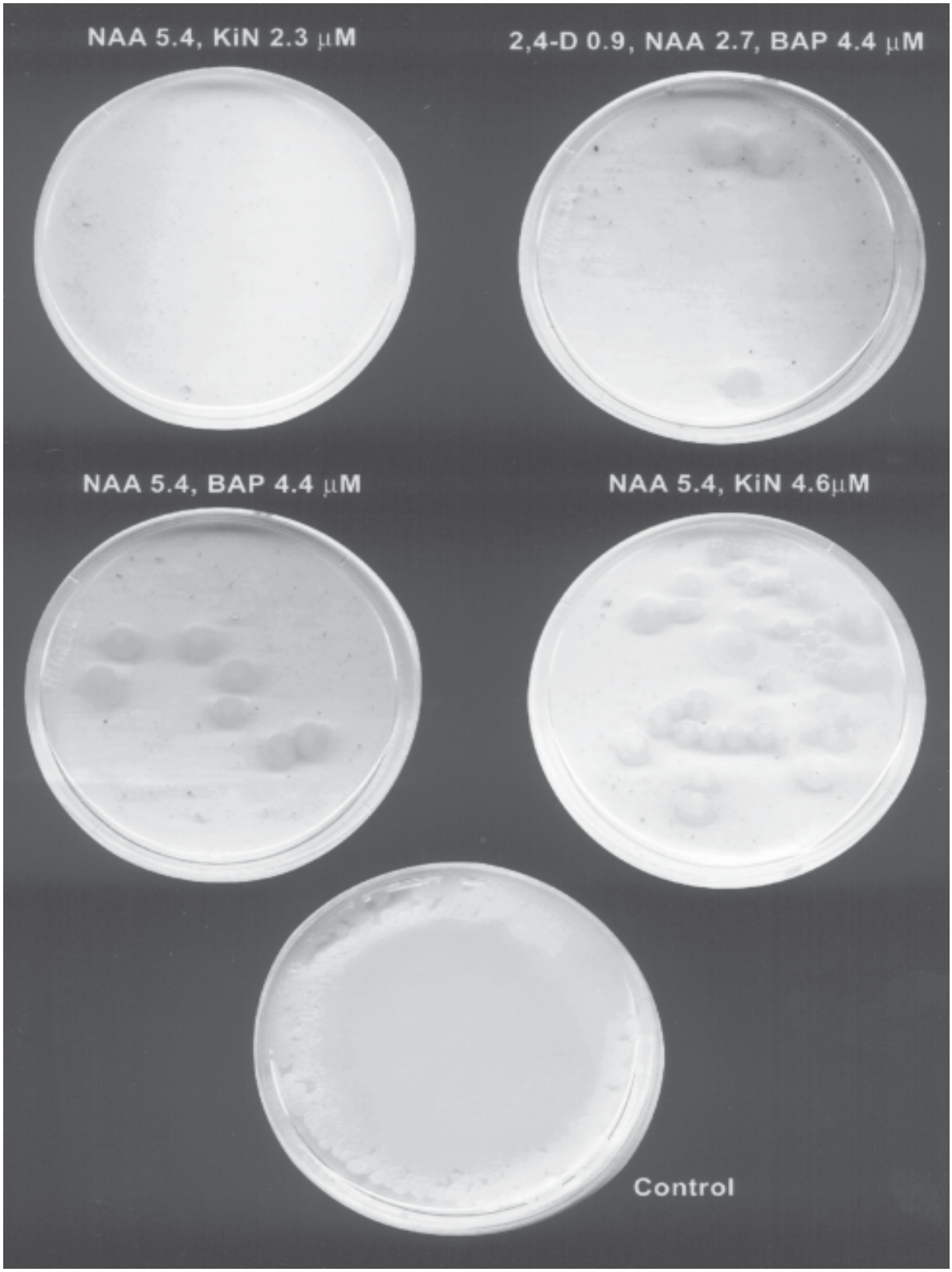

Plate 1. Antibacterial activity of callus extract against *B. subtilis*

Table 2. Anti Bacillus subtilis activity of standard monophenol (dichlorophenol) and diphenol (pyrocatechol)

No.	Dichlorophenol (µg/ml)	Number of colonies with monophenol	Pyrocatechol (µg/ml)	Number of colonies with monophenol
1	2.5	56	0.050	55
2	5.0	48	0.075	43
3	10.0	15	0.100	72
4	15.0	0	0.150	100

of monophenols and diphenols for antimicrobial activities against *B. subtilis.* When antibacterial activity was tested with standard phenols showed that monophenols were more effective than diphenols. However, monophenols were also higher in all calluses as compare to diphenols. These results suggest that monophenols were more effective as an antibacterial agent.

ACKNOWLEDGEMENT

Authors are thankful to Vimal Research Society for Agro-Biotech and Cosmic Powers for providing necessary facilities.

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