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EFFECT OF SOME ANTIMICROBIAL AGENTS ON DIRECT SHOOT ORGANOGENESIS IN *Oxalis corniculata* (L.)

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ABSTRACT

The present study focused on the effect of antimicrobial agents on direct shoot organogenesis in *Oxalis corniculata* Linn. The antimicrobial agents evaluated were kanamycin, carbendazim, cefotaxime and silver thiosulphate. The nodal explants, collected from 4-week old shoots of field grown *O. corniculata* plants, were sterilized by 5% (v/v) Tween-20, followed by 70% ethanol. MS medium was supplemented with alone or in combinations of benzyl aminopurine (BAP), kinetin, naphthalene acetic acid (NAA), indole-3-butyric acid and indole-3-acetic acid. Among the antimicrobial agents tested, carbendazim @ 2 mg L^{-1} along with BAP @ 1 mg L^{-1} gave maximum shooting response (80%), higher shoot number (7.8) and longer shoots (4.7 cm); whereas kanamycin (40 µM) showed least response on shooting (75%) and shoot number (4.5). Among the auxins tested, NAA @ 2 mg L^{-1} showed maximum rooting response (95%), root number (22.3) and root length (4.4 cm). Rooted shootlets were successfully hardened in sterile soil + vermiculite (1:1) medium and then transferred to the field. Among the antimicrobial agents tested, carbendazim + BAP resulted in best response on shooting while NAA showed maximum rooting response.

Keywords: Antimicrobial agents, carbendazim, silver thiosulphate, direct organogenesis, *Oxalis corniculata*

INTRODUCTION

Oxalis corniculata Linn. (creeping wood sorrel or sleeping beauty) is an important medicinal herb. It is a very delicate low-growing herb commonly found in tropical and sub-tropical regions of the world. It is used to treat influenza, fever, urinary tract infections, diarrhoea, sprains and poisonous snakebites (Arya, 1995). The herb is good source of vitamin C and shows antibacterial activity (Unni *et al*., 2009).

Antimicrobial agents are typically included in plant cultures to assess their ability to inhibit or prevent any microbial growth in explants. These agents are usually phytotoxic so are capable to alter the behaviour of cultured plant tissues (Tang *et al*., 2018). Many antimicrobial agents reportedly affect *in vitro* cell culture and plant regeneration (Qin *et al*., 2011). The effect of carbendazim, cefotaxime, kanamycin and silver thiosulphate (STS) on *in vitro* regeneration have been studied in several plants like *Solanum viarum* (Mahadev *et al*., 2014), *Asclepias curassavica (*Salla *et al.*, 2012), *Centella asiatica* (Panathula *et al.*, 2014), however no such studies have been conducted in *Oxalis corniculata.* Carbendazim, fenbendazole and imazalil exhibit fungicidal activity but at the same time are less toxic to the plants (Babu *et al*., 2022). The effects of antimicrobial agents like ampicillin, carbenicillin and cefotaxime greatly vary in different types of explants and even within the same plant species (Gerszberg and Grzegorczyk-Karolak, 2019).

When a plant is wounded or put under environmental stresses or attacked by pathogens, the plant responds through ethylene production. *Brassica campestris* cultured *in vitro* caused abnormal plant

growth and development due to ethylene production (Raghuwanshi and Prasad, 2018). Silver thio-sulphate complex is an inhibitor of ethylene biosynthesis which dissociates in the plant cells as free Ag^{2+} ions. This ion act as anti-ethylene agent (Sreelekshmi, *et al*., 2021). In citrus fruits, ethylene inhibition by silver thio-sulphate causes increase in chlorophyll content and also expands leaf area. However, such studies on *in vitro* plant regeneration of various plant species involving different antimicrobial agents and ethylene inhibitors are very limited (Tung *et al*., 2021). The present study was aimed to assess the effects of some antimicrobial agents and silver thiosulphate on *in vitro* propagation of *O. corniculata*.

MATERIALS AND METHODS

Plant material

The experiments were conducted in the year 2019 to 2020 at the Department of Biotechnology, School of Herbal Studies and Naturo-Sciences, Dravidian University, Kppam, Andhra Pradesh, India. *Oxalis corniculata* was collected from the Herbal Garden, Dravidian University, Kuppam, Andhra Pradesh, India. Axillary bud (0.8 - 1.0 cm in height) explants, excised from 4-week old shoots of field grown plants, were used in present study.

Preparation and sterilization of antimicrobial agents and axillary bud explants

Axillary bud explants were sterilized by using 5% (v/v) tween-20, followed by 70% ethanol. Carbendazim (BASF, India), cefotaxime and kanamycin (HiMedia, India) were filter-sterilized using 0.20 µm filters. Different concentrations of carbendazim (50-300 mg L^{-1}), cefotaxime, silver thiosulphate (STS) and kanamycin (5-100 µM L^{-1}) were prepared. Murashige and Skoog medium (Murashige and Skoog, 1962), devoid of phyto-hormones but containing 3% sucrose, was adjusted to pH 5.8 before adding agar 0.8% (w/v) and then autoclaved. The MS medium was separately supplemented with carbendazim, cefotaxime, kanamycin and STS and axillary buds were inoculated onto the medium. The MS medium without antimicrobial agents and with PGR's served as control. The culture tubes were incubated at 24 ± 2°C under 16 h white light (from white cool florescent tubes at irradiance of 50 µE m^{-2} s^{-1}) and 8 h dark period. Each treatment had 10 replications of explants. The data was subject to the analysis of variance (ANOVA) using SPSS software version 16.0.

Rooting and acclimatization

After regeneration and sufficient elongation, the micro-shoots were carefully excised and rooted on MS medium with IBA, IAA and NAA separately in the concentrations range of 0.5-3.0 mg L^{-1}. Rooted plantlets were transferred to the polycups containing sterile soil and vermiculite (1:1) and covered with plastic bag to maintain humidity. After a month the plantlets were transferred to the greenhouse and planted in soil. Each treatment consisted of ten replicates and the experiment was repeated thrice. All the data were subjected to ANOVA test using SPSS software version 16.0.

RESULTS AND DISCUSSION

Effect of antimicrobial agents on direct shoot organogenesis of nodal explants of O. corniculata

Carbendazim: Efficiency of *in vitro* shoot regeneration depends on many factors such as type of explant, genotype of plant species and the nature of antimicrobial agent supplemented to the medium. The regeneration frequency was improved by the inclusion of antibiotics in the medium formulation. Nodal bud explants were cultured on MS medium supplemented with different concentrations of

carbendazim 50 - 300 mg L^{-1} (Table 1). The antimicrobial agent carbendazim proved more effective in terms of regeneration frequency, shoot number and shoot length. Amongst the carbendazim concentrations tested alone, the highest number of shoots i.e. 6.2 ± 0.02 with shoot length of 4.9 cm and regeneration frequency of 80% was observed in carbendazim 200 mg L^{-1}. The increased shoot number of 7.8 ± 0.01 with mean shoot length of 4.7 cm was observed in treatment carbendazim 150 mg L^{-1} + BAP 1.0 mg L^{-1} (Fig. 1A).

Table 1: The effect of carbendazim alone and in combination with plant growth regulators on *in vitro* shoot organogenesis from nodal explants of *O. corniculata*

Carbendazim (mg L^{-1})	BAP mg L^{-1}	Kn mg L^{-1}	Shooting (%)	No. of shoots explants^{-1} (mean ± SE)	Shoot length (cm) (mean ± SE)
0 (Control - 1)	1.0	-	68	3.0 ± 0.05^{a}	4.1 ± 0.1^{b}
0 (Control - 2)	-	1.0	66	3.1 ± 0.05^{a}	4.2 ± 0.1^{b}
50	-	-	69	3.2 ± 0.05^{a}	4.4 ± 0.1^{b}
100	-	-	73	4.4 ± 0.05^{b}	4.7 ± 0.1^{c}
150	-	-	77	5.5 ± 0.05^{c}	5.5 ± 0.2^{d}
200	-	-	80	6.2 ± 0.02^{e}	4.9 ± 0.1^{c}
250	-	-	72	5.9 ± 0.02^{d}	4.3 ± 0.1^{b}
300	-	-	65	5.3 ± 0.10^{c}	3.3 ± 0.1^{a}
200	1.0	-	80	7.8 ± 0.01^{g}	4.7 ± 0.1^{c}
200	-	1.0	78	6.5 ± 0.05^{f}	5.4 ± 0.1^{d}

Cotrol-1 and -2 explants were grown on MS medium supplemented with phytohormone BAP (mg L^{-1}) and Kn (mg L^{-1}), respectively, but without carbendazim. Observations were recorded after 4 week's growth; The values are the mean ± SE of 10 independent determinations. Data within the same column followed by the same letter indicate no significance at 5% level.

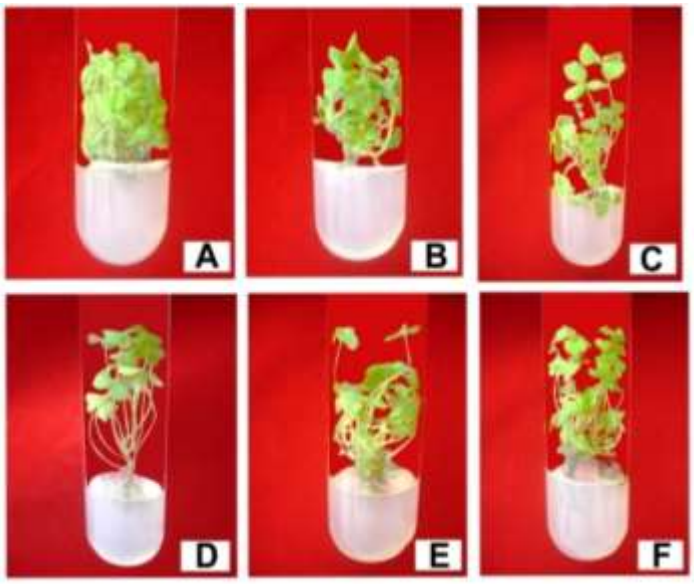

Fig. 1: Direct shoot organogenesis from nodal bud explants on MS medium supplemented with different concentrations of anti-microbial agents. A) Shoot initiation on MS + carbendazim (200 µM); B) Shoot multiplication on MS + carbendazim (200 µM); C) Shoot induction on MS + Cefotaxime (40 µM); D) Shoot multiplication on MS + Cefotaxime (60 µM); E) Shoot initiation on MS + kanamycin (20 µM); and F) shoot multiplication on MS + kanamycin (40 µM).

Carbendazim has much stronger cytokinin-like activities and promotes shoot bud regeneration. Lakshmi *et al*. (2021) reported similar finding on *Ruellia tuberosa*. Carbendazim, extensively used in agriculture, is an organic fungicide that belongs to benzimidazole group and exhibits systemic action. Methyl benzimidazole carbamate or carbendazim has structural similarity to kinetin, adenine and many other adenine-based cytokinins. It has greater fungicidal activity and less toxic effect on plant cells (Sharma *et al*., 2022). Inclusion of carbendazim to control the fungal contamination does not show any cynical effects on *O. corniculata* and further promotes shoot regeneration.

Cefotaxime: Cefotaxime belongs to β-lactum group of antibiotics and shows least toxicity to most plant tissues. It is widely used as a broad spectrum antibiotic against both Gram +ve and Gram –ve bacteria. The assessment of cefotaxime on the regeneration of *O. corniculata* revealed highest number of shoots (5.4 ± 0.05) with regeneration frequency (78%) was obtained at (60 µM) cefotaxime and maximum shoot length (4.2 cm) at 40 µM cefotaxime (Fig. 1C, Table 2). Similar reports were made on *Ruellia tuberosa* (Lakshmi *et al*., 2021). It has been reported in *Centella asiatica* that the cefotaxime showed better elongation and multiplication of shoot (Panathula *et al*., 2014).

Table 2: Effect of cefotaxime added to MS medium on *in vitro* shoot regeneration of plantlets from nodal bud explants of *O. corniculata*

Cefotaxime (µM)	Frequency of regeneration (%)	Mean shoot No. explant^{-1}	Mean shoot length (cm)
Control	64	3.1 ± 0.1^{b}	3.3 ± 0.1^{a}
5	65	3.1 ± 0.1^{b}	3.3 ± 0.1^{a}
10	68	3.4 ± 0.05^{c}	3.6 ± 0.1^{b}
20	70	4.0 ± 0.1^{d}	3.8 ± 0.1^{bc}
40	74	4.7 ± 0.1^{e}	4.2 ± 0.05^{d}
60	78	5.4 ± 0.05^{f}	3.9 ± 0.05^{c}
80	69	3.2 ± 0.05^{b}	3.2 ± 0.2^{a}
100	62	2.8 ± 0.05^{a}	3.1 ± 0.05^{a}

Control is MS medium without cefotaxime. Observations taken after 4 weeks. The values are mean ± SE of 10 independent determinations. Data represent treatment mean ± SE followed by different letter(s) within a column indicate significant differences as per ANOVA and DMRT test (P<0.05).

Kanamycin: The impact of kanamycin on *in vitro* shoot regeneration of *O. corniculata* was analysed. The data revealed that kanamycin influenced the axillary bud cultures of *O. Corniculata* and produced maximum number of shoots (4.5±0.05) with regeneration frequency (75%). Maximum shoot length (5.2 cm) was obtained at40 µM kanamycin (Fig. 1E, Table 3). The regeneration frequencies and shoot number gradually diminished with increase in concentration of kanamycin above 50 µM. Similar findings were reported in apple by Verma *et al*. (2023). The possible mechanism of stimulatory effect of antibiotics on shoot regeneration may involve the generation of stress which makes the cell competent for regeneration; alternatively, these antibiotics may emulate like plant growth regulator (Holford and New bury, 1992).

Table 3: Influence of kanamycin added to MS medium on *in vitro* regeneration of plantlets from nodal explants of *O. corniculata*

Kanamycin (µM)	Frequency of regeneration (%)	Mean shoots explant^{-1} (No.)	Mean shoot length (cm)
Control	63	2.9 ± 0.05^{b}	2.7 ± 0.1^{a}
5	66	3.2 ± 0.05^{b}	2.9 ± 0.1^{a}
10	69	3.8 ± 0.05^{c}	3.1 ± 0.05^{b}
20	71	4.1 ± 0.2^{d}	4.4 ± 0.1^{d}
40	75	4.5 ± 0.05^{e}	5.2 ± 0.05^{f}
60	73	4.2 ± 0.1^{de}	4.9 ± 0.1^{e}
80	62	3.6 ± 0.2^{c}	3.4 ± 0.1^{e}
100	54	2.9 ± 0.05^{a}	3.2 ± 0.05^{b}

Control is grown on MS medium without Kanamycin. Observations taken after 4 weeks; The values are mean ± SE of 10 independent determinations. The treatment means followed by different letter(s) within a column indicate significant differences as per ANOVA and DMRT test (P<0.05).

Silver thiosulphate (STS): The influence of STS (10-40 µM) on *in vitro* shoot regeneration was accomplished by using nodal explants of *O. corniculata* (Fig. 2, Table 4). Shoot bud initiation was observed 1 week after inoculation. The highest number of shoots (5.9 ± 0.02) and regeneration frequency (85%) was obtained at 40 µM (Fig. 2C, E & F) and maximum shoot length (5.7 cm) at 60 µM of STS (Fig. 2D). Ethylene inhibitors promote *in vitro* shoot regeneration in several plant species (Saha and Dutta Gupta, 2018). Silver nitrate is most commonly used as a source of silver ion which can

impede ethylene incorporation at its receptor sites (Beyer, 1979). The Ag^{2+} ions in STS supress ethylene production in explants. The impact of silver ions has been widely reported (Ricci *et al*., 2020).

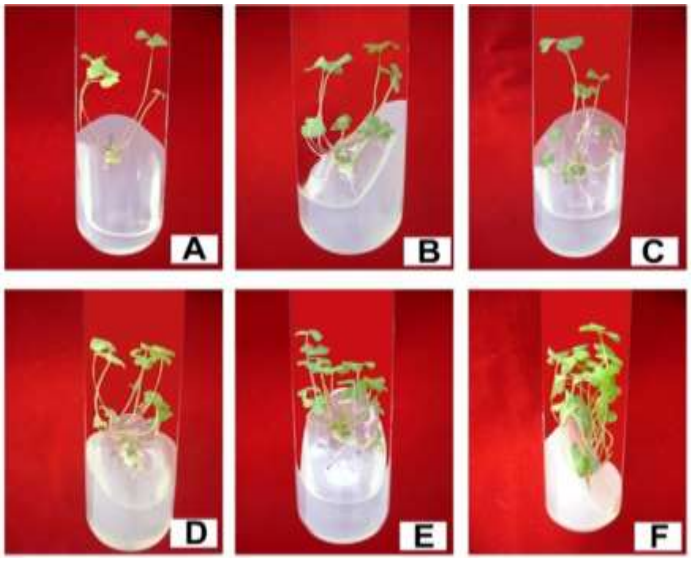

Fig. 2: Direct shoot initiation from nodal explants on MS medium supplemented with different concentrations of silver thio-sulphate (STS). A) STS 10 µM; B) STS 20 µM; C) STS 40 µM; D) STS 60 µM; E) Multiplication of shoots from nodal explants on MS + STS (40 µM); F) Multiplication and elongation of shoots on MS + STS (40 µM).

Table 4: Effect of ethylene antagonist silver thiosulphate (STS) on *in vitro* regeneration of plantlets from nodal bud explants of *O. corniculata*

STS (µM)	Shooting (%)	Mean shoots explant^{-1} (No.)	Mean shoot length (cm)
control	60	1.4 ± 0.05^{a}	3.8 ± 0.1^{a}
5	62	1.4 ± 0.05^{a}	3.8 ± 0.1^{a}
10	65	1.8 ± 0.05^{b}	3.9 ± 0.05^{a}
20	79	2.9 ± 0.05^{d}	4.5 ± 0.1^{bc}
40	85	5.9 ± 0.02^{f}	4.7 ± 0.1^{cd}
60	80	3.2 ± 0.02^{e}	5.7 ± 0.05^{e}
80	75	2.4 ± 0.01^{c}	4.4 ± 0.20^{b}
100	68	1.4 ± 0.05^{a}	4.9 ± 0.05^{d}

Control is grown on MS medium without STS. Observations**:** After 4 weeks; Values are mean ± SE of 10 independent determinations. Data represent treatment means ± SE followed by different letter (s) within a column indicate significant differences according to ANOVA and DMRT test ($P<0.05$).

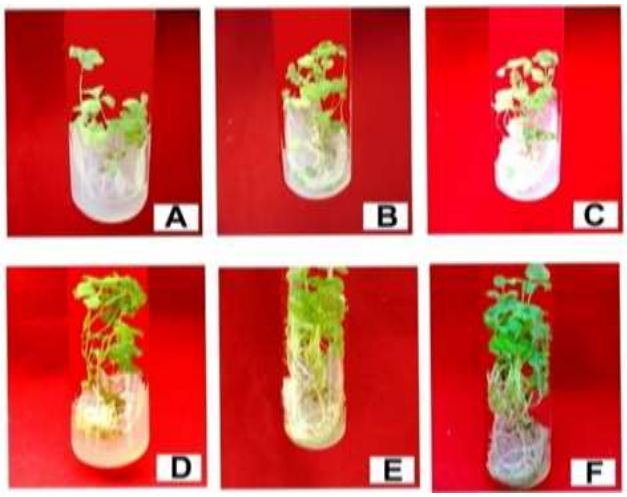

Fig. 3: *In vitro* rhizogenesis from regenerated shoots A) Rhizogenesis on MS + IAA (2.0 mg L^{-1}), B) Rhizogenesis on MS + IAA (1.0 mg L^{-1}), C) Rhizogenesis on MS + IBA (2.0 mg L^{-1}), D) Rooting on MS + IBA (1.0 mg L^{-1}), E) Rooting on MS + NAA (1.0 mg L^{-1}), and F) Rooting on MS + NAA (2.0 mg L^{-1})

In vitro root organogenesis

The shoots derived *in vitro* (3.0-5.0 cm in height) were excised from shoot clump and transferred to the MS medium amended with variable concentrations of auxins such as NAA, IBA and IAA (Fig. 3, Table 5). Among all the concentrations tested, the highest number of roots (22.3 ± 0.1) was found at NAA (2.0 mg L^{-1}) with regeneration frequency of 95% (Table 5, Fig. 3F) whereas the maximum root length (6.5 cm) was achieved at the concentration of NAA (1.0 mg L^{-1}) (Fig. 3E) with regeneration frequency of 93% and the results obtained are consistent with *Ficus religiosa* (Hesami and Daneshvar, 2018). In contrast to our results, MS medium augmented with IBA reported maximum number of roots in banana (Mekonen *et al*., 2021).

Table 5: Rhizogenesis of *in vitro* derived shootlets on MS full strength medium augumented with various concentrations of auxins such as NAA, IBA and IAA

Plant growth regulators (mg L^{-1})			Rooting (%)	Roots explant^{-1} (No.) (mean ± SE)	Root length (cm) (mean ± SE)
NAA	IBA	IAA			
Control	-	-	68	9.1 ± 0.1^{a}	2.5 ± 0.1^{a}
0.5	-	-	90	18.3 ± 0.2^{g}	5.4 ± 0.05^{e}
1.0	-	-	93	20.2 ± 0.4^{h}	6.5 ± 0.3^{f}
2.0	-	-	95	22.3 ± 0.1^{i}	4.4 ± 0.2^{d}
3.0	-	-	89	16.4 ± 0.1^{f}	3.2 ± 0.2^{c}
-	0.5	-	82	12.3 ± 0.3^{d}	3.2 ± 0.05^{bc}
-	1.0	-	87	14.6 ± 0.1^{e}	4.5 ± 0.05^{d}
-	2.0	-	91	16.4 ± 0.1^{f}	5.3 ± 0.1^{e}
-	3.0	-	85	14.4 ± 0.05^{e}	3.3 ± 0.1^{c}
-	-	0.5	72	10.4 ± 0.05^{b}	2.9 ± 0.05^{ab}
-	-	1.0	75	11.8 ± 0.1^{c}	3.4 ± 0.05^{c}
-	-	2.0	77	12.3 ± 0.1^{d}	4.3 ± 0.2^{d}
-	-	3.0	69	9.5 ± 0.1^{a}	2.7 ± 0.1^{a}

Control is grown on MS medium without phytohormones. Observations made after 4 weeks; Values are mean ± SE of 10 independent determinations. Data within the same column followed by the same letter indicated no significance at 5% level.

In MS medium supplemented with IBA (0.5 - 3.0 mg L^{-1}), the maximum number of roots (16.4 ± 0.1) was observed at IBA concentration of 2.0 mg L^{-1} with 91% regeneration frequency (Fig. 3C; Table 5). The efficacy of IBA in rooting has earlier been documented in pomegranate (Mehta *et al*., 2018). But the number of shoots was less when compared to NAA. This was followed by IAA (2.0 mg L^{-1}) has produced the maximum shoot number (12.3 ± 0.1) with regeneration frequency of 77% (Fig. 3A). Effect of IAA on rooting for continued existence of the regenerated plantlets upon transfer to soil under natural conditions. The healthy rooted plantlets of *O. corniculata* were transferred to tray containing autoclaved soil and vermiculite in 1:1 ratio under high relative moisture (60 - 70%) for acclimatization and permitted to cultivate under nursery shade conditions. The plantlets were watered regularly at two days gap and were at last planted in field conditions where exhibited survival rate of 75% (Fig. 4). Acclimatization of the regenerated plants to the outside environment is a last phase of micropropagation and the success relies on upon many factors (Isah, 2015). Several researchers reported the superior growth and yield of the *in vitro* regenerants compared to *in vivo* plants (Yücesan *et al*., 2016).

Fig. 4: Acclimatization of *in vitro* raised plantlets. A) polythene bag containing soil and vermiculite (1:1); B) Mass propagation of tissue cultured plants in earthen pot and finally transferred to field conditions

Conclusion: Among the antimicrobial agents studied, carbendazim with BAP resulted best response on shooting and NAA has given maximum rooting response in *O. corniculata*.

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