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Elimination of '*Candidatus Phytoplasma asteris*' from rose (*Rosa* × *hybrida* L.) by application of antibiotics under *in-vitro* and *in-vivo* conditions

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ABSTRACT

Rose is severely affected by phytoplasma diseases, causing significant loss in flower quality. A valid control strategy is not yet available for managing phytoplasma diseases in roses. The present study aimed to obtain phytoplasma-free rose plants using antibiotics. In this study, phytoplasma-associated rose cultivar 'Dr. M. S. Randhawa' exhibiting phyllody and flower malformation symptoms was used to study the effect of three antibiotics namely, oxytetracycline, streptomycin and erythromycin A at different concentrations (60 mg/L, 80 mg/L and 100 mg/L) for management of phytoplasma under *in-vitro* and *in-vivo* conditions. All concentrations of oxytetracycline effectively eliminated phytoplasma in both conditions. The absence of phytoplasma was confirmed by nested Polymerase Chain Reaction (PCR) analysis in all treated plants *in-vivo* and *in-vitro* at monthly intervals for up to three months. However, streptomycin and erythromycin A treatments failed to eliminate phytoplasma in both conditions, as indicated by positive PCR results. The findings of this study reveal that oxytetracycline at 60 mg/L was the most efficient in eliminating phytoplasma from infected rose plants under both *in-vitro* and *in-vivo* conditions.

Key words: Phyllody, oxytetracycline, 16S rRNA, 23S rRNA peptidyl transferase loop.

INTRODUCTION

Rose (*Rosa* × *hybrida* L.), universally acclaimed as the "Queen of flowers," is one of the most commercially significant cut flowers, holding the top rank in both domestic and international trade. In addition, roses are highly valued and extensively used as loose flowers, pot plants, and garden plants. They are also an important source of rose water, essential oils, and several value-added products used in the perfumery industry (Raju *et al*., 11). The total area under rose cultivation in India is 29.41 thousand hectares, with a production of 301.95 thousand metric tons (NHB, 9).

Phytoplasmas (*Candidatus Phytoplasma*) are plant pathogens belonging to the bacterial class *Mollicutes*. They lack rigid cell walls and are spherical or pleiomorphic in shape, with sizes similar to those of mycoplasmas (80-800 nm). Phytoplasmas are transmitted by plant hoppers, leafhoppers, and psyllids (Hogenhout *et al*., 7; Rao, 12; Rihne *et al*., 15). They are associated with various symptoms such as phyllody, yellows, virescence, witches' broom, shoot proliferation, little leaf, stunting, flower malformations, and fasciation in approximately ninety species of ornamental plants. These have been characterized and classified into fourteen 16S ribosomal phytoplasma groups worldwide (Bellardi *et al*., 2). In India, more than 40 ornamental plants have been found to be associated with six different ribosomal groups (Rao *et al*., 13). Rose plants are also infected by different groups of phytoplasmas, exhibiting a wide range of specific and non-specific symptoms in foliage and flowers. Phytoplasma infection alters flower morphology, reducing its aesthetic appeal and marketability. Eliminating phytoplasma remains a challenge, as infected plants cannot be completely healed. Since, the biological cycle involves both plant hosts and insect vectors, conventional control strategies primarily focus on eradicating infected plants and applying insecticides against vectors. However, these approaches have significant economic and environmental impacts (Bianco *et al*., 4). Phytoplasma elimination techniques attempted earlier include *in-vitro* and *in-vivo* thermotherapy, shoot tip culture (Laimer and Bertaccini, 8), antibiotic treatments (Chiesa *et al*., 5; Tanno *et al*., 17), cryotherapy, and the induction of plant resistance through abiotic or biotic inducers (Ustun *et al*., 19).

Given the commercial importance of roses and the limited research on phytoplasma management in this crop, the present study aimed to mitigate or control phytoplasma infection in rose cv. 'Dr. M.S. Randhawa,' associated with *Candidatus Phytoplasma asteris*, through treatment with three different antibiotics-oxytetracycline, streptomycin, and erythromycin under both *in-vitro* and *in-vivo* conditions.

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MATERIALS AND METHODS

Phytoplasma suspected symptoms such as phyllody and flower malformation were observed in rose cv. 'Dr. M.S. Randhawa' at the experimental field of Division of Floriculture and Landscaping, ICAR-IARI, New Delhi. The samples were collected and analyzed for phytoplasma identification by Polymerase Chain Reaction (PCR) assays and sequencing in our previous study (Rihne *et al.*, 14). Infected and non-symptomatic plants were further used for *in-vitro* and *in-vivo* management studies. To test the efficacy of antibiotics in management of phytoplasma in rose, three antibiotics, *viz*., oxytetracycline, streptomycin and erythromycin A of varying concentration (60, 80 and 100 mg/L) were used in both *in-vitro* and *in-vivo* conditions in the present study. Nodal segments (2-3 cm) with axillary buds were isolated from phytoplasma-associated rose cv. 'Dr. M.S. Randhawa' and kept under running water for 30 minutes. Explants were then treated with 0.2% carbendazim, 0.2% metalaxyl-M, 200 ppm HQC, and 200 ppm citric acid, followed by surface sterilization with 1% sodium hypochlorite for 2 min and three washes with autoclaved double-distilled water in a laminar airflow chamber. Pre-treated explants were aseptically inoculated on MS medium supplemented with 5 ml/L BAP, 0.2 ml/L NAA and 0.5 ml/L GA_3 (Fig. 1a), along with filter-sterilized antibiotics—oxytetracycline, streptomycin, and erythromycin A at 60, 80, and 100 mg/L. Control explants were cultured without antibiotics. Explants on antibiotic-containing medium were subcultured onto antibiotic-free MS medium with 5 ml/L BAP (Fig. 1b) every 15 day. Cultures were maintained for six months, with periodic subculturing, until sufficient growth was achieved for PCR analysis to detect phytoplasma presence. Shoot length, bud sprouting time, and bud sprouting percentage were recorded, and data were analyzed using a completely randomized design with Arc Sin √% transformation before ANOVA.

Nodal segments from phytoplasma-infected rose (GenBank Acc. No. MW309814) were used for *in-vitro* mass multiplication. Subculturing was performed every 15 days for 3-4 months until sufficient plantlets developed. These were rooted in MS medium with rooting hormones and acclimatized before transfer to pots under a shade net house. PCR assays confirmed phytoplasma presence in regenerated plants, which were then used for *in-vivo* studies. For *in-vivo* management, treatments included control (no antibiotics) and foliar sprays of oxytetracycline, streptomycin, and erythromycin A at 60, 80, and 100 mg/L, applied twice at 15-day intervals. The potting mixture comprised equal parts peat and perlite, with

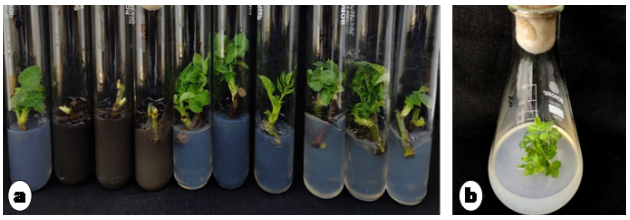

Fig. 1. *In-vitro* antibiotics treatment: a) cultures after 15 days of inoculation in medium containing antibiotics and b) transfer of explants onto antibiotic-free medium.

no fertilizers. Treated plants were observed for 45 days, and greenhouse-grown plants were tested for phytoplasma presence (Fig. 2).

DNA was extracted using the DNASure® plant DNA extraction kit (Genetix Biotech Asia Pvt. Ltd.). PCR assays used phytoplasma-specific primers (P1/P7, R16mF2/R16mR2, and R16F2n/R16R2) in a nested PCR assay (Rihne *et al*., 14). PCR cycling conditions included 35 cycles of denaturation (94°C, 45 s), annealing (55-56°C, 1 min), and extension (72°C, 2 min), with a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1% agarose gel, stained with GoodView™ nucleic acid stain, and visualized using a UV transilluminator (Azure 200 Gel documentation system, USA).

RESULTS AND DISCUSSION

Severe disease incidence of 87.5% was recorded in cv. Dr. M.S. Randhawa exhibiting phytoplasma symptoms of phyllody, virescence and flower malformation at research farm of ICAR-IARI, New Delhi. In our previous study, rose cv. 'Dr. M.S. Randhawa' phytoplasma strain was identified as member of 16SrI-B and 16SrII-D subgroup on the basis of BLASTn, phylogeny and *in silico* RFLP

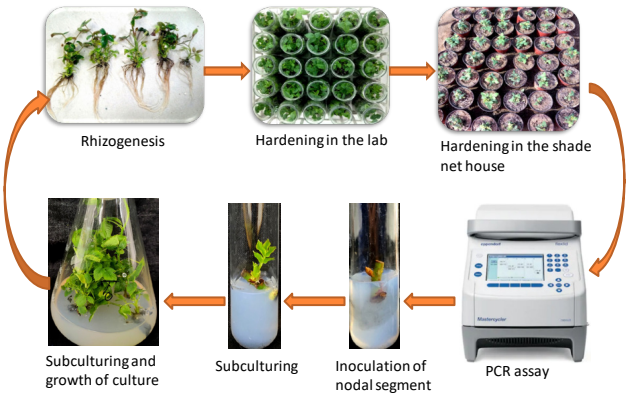

Fig. 2. Cycle showing molecular detection of phytoplasma and *in-vitro* plant regeneration for *in-vivo* management of phytoplasma.

analysis by using pDRAW32 software developed by AcaClone Software (http://www.acaclone.com) (Rihne *et al.*, 15). Management of phytoplasma disease associated with rose cv. 'Dr. M.S. Randhawa' was attempted *in-vitro* and *in-vivo* using oxytetracycline, streptomycin, and erythromycin A at three different concentrations (60, 80 and 100 mg/L).

Under *in-vitro* conditions, phytoplasma remission was noticed in oxytetracycline-treated plantlets, as evidenced by the lack of amplification of the 16S rRNA phytoplasmal gene in all oxytetracycline (60, 80 and 100 mg/L) treated culture media in nested PCR assays (Fig. 3). Oxytetracycline rose treated plantlets showed significant growth retardation in shoot length of 8.00 mm in T_1 (60 mg/L), 5.30 mm T_2 (80 mg/L), 4.30 mm T_3 (100 mg/L) after fifteen days of inoculation as compared to control (25.00 mm shoot length). Bud sprout was recorded the maximum of 22.00 days in T_3 followed by 18.60 days in T_2 and 17.30 days in T_1. However, bud sprouting percentage was recorded only after thirty days of inoculation and was found significantly reduced in oxytetracycline treated T_1 (81.40%), T_2 (77.70%) and T_3 (66.60%) plants (Table 1). The oxytetracycline treated regenerated rose plants regained active/ normal growth after successive sub culturing in antibiotic free media in six months, exhibited high proliferation and the sub cultured plants were tested phytoplasma negative with normal and healthy growth. Streptomycin and erythromycin A treatments failed to show elimination of phytoplasma in the *in-vitro* regenerated rose plantlets as manifested by testing positive for phytoplasmas in PCR assays (Fig. 3). Streptomycin treatments T_4 (60 mg/L), T_5 (80

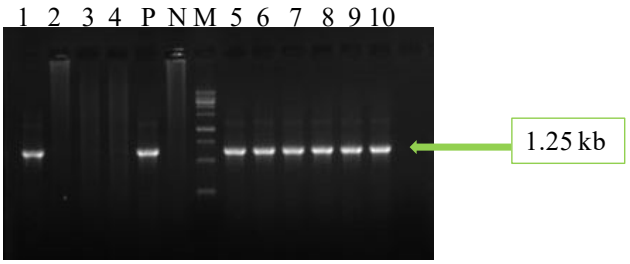

Fig. 3. Phytoplasma indexing of *in-vitro* antibiotic treated plants and gel images showing results of amplifications by different sets of primer pairs: P1/ P7 and R16mF2/R16mR2 and R16F2n/R16R2 lane 1: T_0 (Control), lane 2: T_1 (60 mg/L oxytetracycline), lane 3: T_2 (80 mg/L oxytetracycline), lane 4: T_3 (100 mg/L oxytetracycline), lane 5: T_4 (60 mg/L streptomycin), lane 6: T_5 (80 mg/L streptomycin), lane 7: T_6 (100 mg/L streptomycin), lane 8: T_7 (60 mg/L erythromycin), lane 9: T_8 (80 mg/L erythromycin), lane 10:T_9 (100 mg/L erythromycin), lane M: marker, lane P: Positive control, lane N: negative control.

Table 1. Effect of antibiotics on elimination of *Candidatus Phytoplasma asteris* in rose under *in-vitro* conditions.

Treatment	Antibiotic	Shoot length* (mm)	Bud sprout** (%)	Days taken to bud sprout	Plant growtth	Presence of phytoplasma***
T_0	Control	25.00	96.20 (83.40)	4.30	Normal growth	Phytoplasma positive (+)
T_1	60 mg/L oxytetracycline	8.00	81.40 (68.80)	17.30	Slow growth at initial stages	Phytoplasma negative (-)
T_2	80 mg/L oxytetracycline	5.30	77.70 (61.70)	18.60	Slow growth at initial stages	Phytoplasma negative (-)
T_3	100 mg/L oxytetracycline	4.20	66.6 (54.80)	22.00	Slow growth at initial stages	Phytoplasma negative (-)
T_4	60 mg/L streptomycin	24.00	85.1 (67.50)	7.30	Normal growth	Phytoplasma positive (+)
T_5	80 mg/L streptomycin	24.30	85.1 (67.50)	7.60	Normal growth	Phytoplasma positive (+)
T_6	100 mg/L streptomycin	14.60	70.3 (57.00)	8.00	Normal growth	Phytoplasma positive (+)
T_7	60 mg/L erythromycin	24.30	92.5 (76.90)	8.00	Normal growth	Phytoplasma positive (+)
T_8	80 mg/L erythromycin	14.30	92.5 (76.90)	6.00	Normal growth	Phytoplasma positive (+)
T_9	100 mg/L erythromycin	14.30	88.8 (74.00)	7.30	Normal growth	Phytoplasma positive (+)
SE(m)		0.67	5.12 (5.93)	1.53		
$CD_{(0.05)}$		1.97	15.20 (17.61)	4.57		

*The shoot length was measured 15 days after inoculation
**Bud sprout percentage was recorded 30 days after inoculation
***Growth and PCR assays detection to confirm presence/absence of phytoplasm as was observed after six months of antibiotics application

mg/L) and T_6 (100 mg/L) failed to show elimination of phytoplasma in the *in-vitro* regenerated rose plantlets as manifested by testing positive for phytoplasma in PCR assays (Fig. 3). The inoculated explant took longer time in bud sprouting in T_4 (7.30 days), T_5 (7.60 days) and T_6 (8.00 days) as compared to control but the days taken to sprout was lesser compared to oxytetracycline treated plantlets (Table 1). In streptomycin treated regenerated plants, significant effect on shoot length was noticed at 100 mg/L (14.60 mm) and bud sprouting (70.30%) as compared to 25.00 mm shoot length and 96.20 % bud sprouting in control (Table 1). However, no significant difference was noticed at 60 and 80 mg/L concentration. Erythromycin A treatments T_7 (60 mg/L), T_8 (80 mg/L), and T_9 (100 mg/L) failed to show elimination of phytoplasma in the *in-vitro* regenerated plantlets, as evidenced by phytoplasma positive PCR results (Fig. 4). In erythromycin A treated regenerated plants, significant effect on shoot length was noticed at 80 mg/L (14.30 mm) and 100 mg/L (14.30 mm) and no significant difference at 60mg/L (24.30 mm) as compared to control (25.00 mm). Days taken to bud sprouting and bud sprouting % also did not show significant difference in the three levels of erythromycin A treatments (Table 1).

Under *in-vivo* conditions, successful elimination of *Ca*. *P. asteris* from rose cv. 'Dr. M.S. Randhawa' was recorded with foliar spray of oxytetracycline at all concentrations, confirmed by no phytoplasma DNA amplification in PCR assay (Fig. 4). However, streptomycin and erythromycin A were ineffective, as phytoplasma 16S rRNA gene amplification was observed in treated samples. Normal growth occurred in plants treated with oxytetracycline at 60 and 80 mg/L, while poor growth was noted at

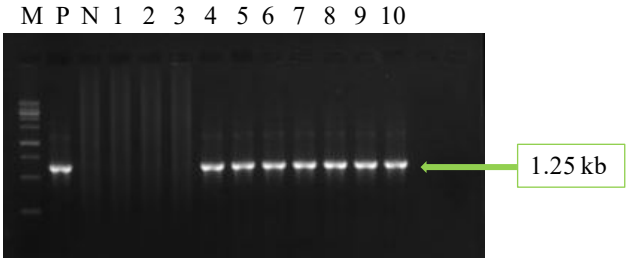

Fig. 4. Phytoplasma indexing of *in-vivo* antibiotic treated plants and gel images showing results of amplifications by different sets of primer pairs: P1/P7 and R16mF2/R16mR2 and R16F2n/R16R2 lane 1: T_1 (60 mg/L oxytetracycline), lane 2: T_2 (80 mg/L oxytetracycline), lane 3: T_3 (100 mg/L oxytetracycline), lane 4: T_0 (Control), lane 5: T_4 (60 mg/L streptomycin), lane 6: T_5 (80 mg/L streptomycin), lane 7: T_6 (100 mg/L streptomycin), lane 8: T_7 (60 mg/L erythromycin A), lane 9: T_8 (80 mg/L erythromycin A), lane 10:T_9 (100 mg/L erythromycin A), lane M: marker, lane P: Positive control, lane N: negative control.

100 mg/L after 45 days. Poor growth was also seen in streptomycin- and erythromycin-treated plants. Control plants exhibited normal growth but retained phytoplasma (Table 2). PCR amplification of phytoplasma 16S rRNA gene was performed using P1/P7 as the first primer pair, followed by nested primers R16mF2/R16mR2 and R16F2n/R16R2. 16SrI-B subgroup phytoplasma strains (GenBank Acc. No. MW309814) yielded ~1.25 kb amplicons in nested PCR. No amplification was detected in asymptomatic rose samples with the same primers (Fig. 4 & 5). Tetracyclines remain the only antibiotics capable of suppressing phytoplasma symptoms and multiplication, though complete eradication remains

Table 2. Response of antibiotic treatment on elimination of *Candidatus Phytoplasma asteris* in rose cv. 'M.S. Randhawa' under *in-vivo* conditions.

Treatment	Antibiotic	Plant growtth	Presence of phytoplasma*
T_0	Control	Normal growth	Phytoplasma positive (+)
T_1	60 mg/L oxytetracycline	Normal growth	Phytoplasma negative (-)
T_2	80 mg/L oxytetracycline	Normal growth	Phytoplasma negative (-)
T_3	100 mg/L oxytetracycline	slow growth	Phytoplasma negative (-)
T_4	60 mg/L streptomycin	poor growth	Phytoplasma positive (+)
T_5	80 mg/L streptomycin	poor growth	Phytoplasma positive (+)
T_6	100 mg/L streptomycin	Very poor growth	Phytoplasma positive (+)
T_7	60 mg/L erythromycin	Poor growth	Phytoplasma positive (+)
T_8	80 mg/L erythromycin	Poor growth	Phytoplasma positive (+)
T_9	100 mg/L erythromycin	Very poor growth	Phytoplasma positive (+)

*PCR assays detection to confirm presence/absence of phytoplasmas after 45 days of antibiotics application.

challenging (Bertaccini, 3). Previously, tetracycline was successfully used to control pear decline, peach rosette, apple proliferation, brinjal little leaf, potato purple top, sesame phyllody, sugarcane grassy shoot disease, and Chrysanthemum phyllody under *in-vitro* and *in-vivo* conditions (Bianco *et al*., 4).

Our study confirms oxytetracycline's efficacy in eliminating phytoplasma under both conditions. Similar results were reported in *Catharanthus roseus* (Madhupriya, 9), *Chrysanthemum morifolium* (Taloh *et al*., 16), *Chrysanthemum coronarium* (Tanno *et al*., 17), *Portulaca grandiflora* (Ajayakumar *et al*., 1), and *Tagetes erecta* (Panda *et al*., 10). Phytoplasma management remains difficult due to its inability to be cultured artificially, variable accumulation in plants, and horizontal transmission *via* insect vectors. Tetracycline targets bacterial 16S rRNA, and resistance mutations in mycoplasmas (Degrange *et al*., 6) have not been observed in phytoplasmas (Tanno *et al*., 17), explaining its efficacy in this study. Erythromycin A and streptomycin, belonging to the macrolide class, were ineffective against phytoplasma in roses. Macrolides, including erythromycin, azithromycin, clarithromycin, and leucomycin, failed to reduce '*Ca. P. asteris*' accumulation despite their toxicity to mycoplasmas (Tanno *et al*., 17). Macrolide-resistant bacteria often have mutations in the 23S rRNA peptidyl transferase loop (Taylor-Robinson and Bebear, 18), and phytoplasmas innately possess resistance bases (Tanno *et al*., 17), likely explaining macrolide ineffectiveness.

Our findings suggest oxytetracycline at 60, 80 and 100 mg/L effectively eliminated phytoplasma under both *in-vitro* and *in-vivo* conditions. Growth delay was minimal at 60 mg/L *in-vitro*, with no distinct effects observed across concentrations *in-vivo*. Given its efficacy at lower concentrations, 60 mg/L oxytetracycline is recommended for managing phytoplasma in roses, reducing costs and environmental impact.

AUTHORS' CONTRIBUTION

Conceptualization and designing of the research work (TR, N, GPR); Execution of field/lab experiments and data collection (TR, N, GPR, MK); Analysis of data and interpretation (TR, N, GPR, AT, KPS); Preparation and finalization of manuscript (TR, N, MK, KPS, AT).

CONFLICT OF INTEREST

The authors have no conflict of interest.

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