Development and validation of marker-free constructs based on nucleocapsid protein gene of watermelon bud necrosis orthotospovirus in watermelon

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Marker-free (MF) constructs were developed using 825 bp and 426 bp *N* genes of watermelon bud necrosis orthotospovirus (WBNV). *N* genes were amplified and cloned in pDrive (825 bp) and/or pGEMT (426 bp) vectors and recombinants were confirmed. pCAMBIA 2300 was restricted with *Xho*I to release *npt*II gene and re-circularized; it is called MF-pCAMBIA 2300. GUS cassette with promoter and terminator from pBI 121 was cloned into the MCS region of MF-pCAMBIA 2300 with *Eco*RI and *Hin*dIII. Eluted PCR products of 825 and 426 bp were cloned in already developed MF-pCAMBIA 2300. These *N* gene constructs were then mobilized in *Agrobacterium tumefaciens* strain EHA 105. Agroinfiltration of *A. tumefaciens* harbouring MF-pCAMBIA 2300 *N* gene constructs was practised by syringe method in 20-day-old watermelon seedlings. PCR amplification showed 825 and 426 bp amplicons which confirmed their presence in agroinfiltrated seedlings. Further, three days of post agroinfiltration seedlings were challenge-inoculated with WBNV and kept under artificial light for 3–6 days. Agroinfiltrated and inoculated seedlings were tested by ELISA and resulted positive with OD ranging from 0.31 to 0.46 and 0.35 to 0.43 in full and partial *N* genes respectively. Six days post-inoculation, necrosis and complete drying of the leaves were observed. The systemic upper young leaves were subjected to DAC-ELISA and results revealed negative reaction with OD ranging from 0.11 to 0.19. The *N* gene constructs conferred resistance against WBNV in watermelon.

Keywords: Agroinfiltration, bud necrosis disease, orthotospovirus, watermelon.

WATERMELON bud necrosis orthotospovirus (WBNV), one of the most emerging and serious pathogens affecting watermelon, continues to cause severe yield loss up to 100% in various cucurbitaceous hosts in India[1,2]. During 1991–92, an unusual disease of watermelon was recorded in the experimental fields of the Indian Institute of Horticultural Research (IIHR), Bangalore[3]. Later, a similar disease was recorded in watermelon-growing states of Karnataka, Andhra Pradesh and Maharashtra and 39–100% disease incidence and 60–100% loss in yield were observed[4]. Causal agent of this disease was identified as a distinct *Orthotospovirus* species designated as WBNV based on the nucleocapsid (N) protein gene sequence information and previously determined host range differences[5]. The amino acid sequence of *N* gene showed identity of about 84% and 82% with corresponding *N* genes of watermelon silver mottle orthotospovirus (WSMoV) and groundnut bud necrosis orthotopsovirus (GBNV) respectively.

WBNV belongs to the genus *Orthotospovirus* (formerly known as *Tospovirus*) and family Tospoviridae (formerly known as Bunyaviridae) having quasi-spherical enveloped virions of 80–110 nm in diameter and has tripartite ssRNA genome segments designated as large (L), medium (M) and small (S) RNAs[6,7]. The S and M-RNA segments are ambisense in orientation. S-RNA encodes for non-structural protein (NSs; 49.6 kDa) in viral sense and nucleocapsid (N; 30 kDa) protein in viral complementary sense having a size of 3.401 kb. Whereas M-RNA encodes for a movement protein (NSm; 34.1–34.22 kDa) in viral sense and a precursor of glycoproteins (Gn and Gc; 127.15–127.5 kDa) in complementary sense having a size of 4.794 kb. L-RNA encodes for RNA dependent RNA polymerase (RdRp) (332 kDa) having genome size of 8.916 kb (refs 8–10).

Managing orthotospoviruses poses a formidable challenge and this has received considerable attention due to their serious economic impact on crop production[2]. Extensive studies have attempted to engineer host resistance using partial or intact *N* gene sequences against orthotospoviruses which led to heritable immunity against homologous virus-species[11–21]. Further, RNAi approach has been demonstrated for development of transgenic against orthotospoviruses[22,23] including broad-spectrum transgenic resistance[24].

To increase the level of natural resistance, transgenic resistance could be energized. First successful *Agrobacterium*-mediated transformation of watermelon with cucumber green mottle mosaic virus CP gene for developing virus-resistant plant was reported[25] with low transformation efficiency. Recently, *Agrobacterium*-mediated plant transformation using *N* gene, transgenic *Citrullus* and other species has been developed against WSMoV, GBNV and WBNV[26–30].

Therefore, an attempt has been made to develop the *N* gene-derived marker-free constructs using pCAMBIA 2300 and is further validated by agroinoculating into the watermelon. This approach is a way forward in understanding the role of *N* gene and its application in further WBNV transgenic development programmes.

Two marker-free constructs (full and partial *N* genes) were developed from already cloned *N* gene of WBNV isolate in pGEMT Easy vector maintained at the Advanced Centre for Plant Virology. Two pairs of *N* gene specific

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primers were designed by incorporating *Bam*HI and *Sac*I sites in forward and reverse primers of full *N* gene respectively (BM01Fa 5′-TT**GGATCC**ATGTCTAACGT AAAGCAGCT-3′ and BM02 R′ 5′-AA**GAGCTC**TTACA CTTCCAAAGAAGTGC3′), whereas *Xho*I was incorporated in both the primers for partial *N* gene, i.e. RKJ162F 5′-CCGGC**CTCGAGT**GGAACTGCCATTGGTG-3′ and RKJ163R 5′-AA**CTCGAG**TTACACTTCCAAAGAAG-TG-3′).

Full *N* gene (825 bp) was amplified from pGEMT easy vector following the PCR reaction. PCR was conducted in a thermo cycler (Biometra, Germany) with the following temperature conditions: 1 min hot start at 94°C followed by 30 cycles of denaturation at 94°C for 30 sec, annealing for 1 min at 52°C and synthesis at 72°C for 1 min, and a cycle of final extension at 72°C for 10 min. The resulting PCR product (825 bp) was analysed using 1% agarose gel electrophoresis. Full *N* gene (825 bp) was cloned in pDrive vector and recombinant colonies were selected by colony PCR and restriction digestion with *Bam*HI and *Sac*I. The 825 bp PCR products were purified using SV-Wizard PCR clean up system (Promega, Madison, USA). The purified PCR product was cloned in marker-free (MF) pCAMBIA 2300. The ligation reaction mixture (20 µl) contained 10 µl of digested and purified insert (full) DNA (~250 ng), 5 µl of digested and purified MF-pCAMBIA 2300 (~100 ng), 2 µl of ligase 10× buffer, 1 µl T4 DNA ligase (Fermentas, USA) and 2 µl of autoclaved water. Ligation mixture was incubated at 16°C overnight. Competent cells of *Escherichia coli*, strain DH5α, were transformed with recombinant clones which were selected by colony PCR as described above. MF-pCAMBIA 2300 was formed by restricting its plasmid with *Xho*I (Fermentas, USA) resulting in the removal of *npt*II kanamycin resistance gene from the vector. Restricted pCAMBIA 2300 was re-circularized and the resulting plasmid was called MF-pCAMBIA 2300. To get the promoter and terminator in MF-pCAMBIA 2300, the GUS cassette containing CaMV 35S promoter and NOS terminator from pBI 121 was cloned into the MCS region of pCAMBIA 2300 MF with *Eco*RI and *Hin*dIII (Fermentas, USA). After insertion of the GUS cassette in MF-pCAMBIA 2300, *GUS* gene was released by *Bam*HI and *Sac*I and the restricted 825 bp full *N* gene with the same restriction enzymes was ligated in its place.

Partial *N* gene (426 bp) was amplified from pGEMT easy vector following the 100 µl PCR reaction. PCR was conducted in a thermo cycler (Biometra, Germany) with similar temperature conditions as full *N* gene except 45 sec hot start at 94°C, annealing for 1 min at 50°C and synthesis at 72°C for 45 sec. The resultant PCR product (426 bp) was analysed using 1% agarose gel electrophoresis.

Partial *N* gene (426 bp) was cloned in pGEMT easy vector and recombinant colonies were selected by colony PCR and restriction digestion with *Xho*I (Fermentas, USA). PCR products (426 bp) were purified as mentioned above. Purified PCR product was cloned in MF-pCAMBIA 2300. Ligation of partial *N* gene in MF-pCAMBIA 2300 was carried out as explained above for full *N* gene.

MF-pCAMBIA 2300 consisting of GUS cassette containing CaMV 35S promoter and NOS terminator from pBI 121 was ligated into the MCS region. MF-pCAMBIA 2300 plasmid was restricted with *Xho*I resulting in the removal of *npt*II kanamycin resistance gene from the vector. Already restricted partial *N* gene (426 bp) with *Xho*I was inserted in place of *npt*II.

Full and partial *N* genes MF-pCAMBIA 2300 viral constructs were mobilized from *E. coli* into *A. tumefaciens* (EHA 105) using freezing and thawing method[31]. The transient assay was done for validation of the constructs based on full and partial *N* genes in watermelon according to the protocol described by Wydro *et al.*[32]. Agroinfiltrated seedlings were kept in a glasshouse. Tissues were harvested from these watermelon seedlings at 3 and 6 days of post-agroinfiltration and subjected to DAC-ELISA for confirmation. Further, 3 days of post-agroinfiltration, these seedlings were challenge-inoculated with WBNV and kept for 3–6 days for symptoms observation and testing by DAC-ELISA and RT-PCR.

Marker-free constructs were developed using complete (825 bp) and partial (426 bp) nucleocapsid (N) protein genes (Figure 1). The complete *N* gene from the pBINAR binary vector was amplified (Figure 2 *a*; lane 1) and cloned in pDrive vector and recombinants showed the amplification of 825 bp amplicons in colony PCR. Similarly, restriction digestion with *Bam*HI and *Sac*I (double digestion) resulted in the release of 825 bp fragment along with inward pDrive (3850 bp) vector in 1% agarose gel electrophoresis (Figure 2 *b*).

Eluted 825 bp product was cloned in already developed MF-pCAMBIA 2300. Colony PCR and restriction digestion

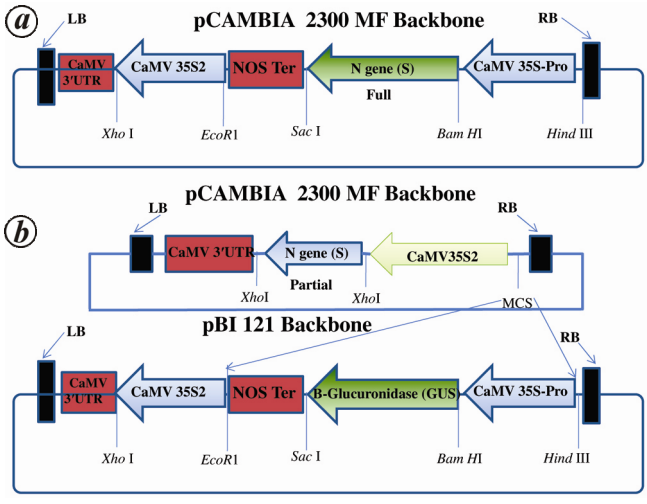

Figure 1. Schematic presentation of the restriction map of complete (***a***) and partial (***b***) *N* gene constructs.

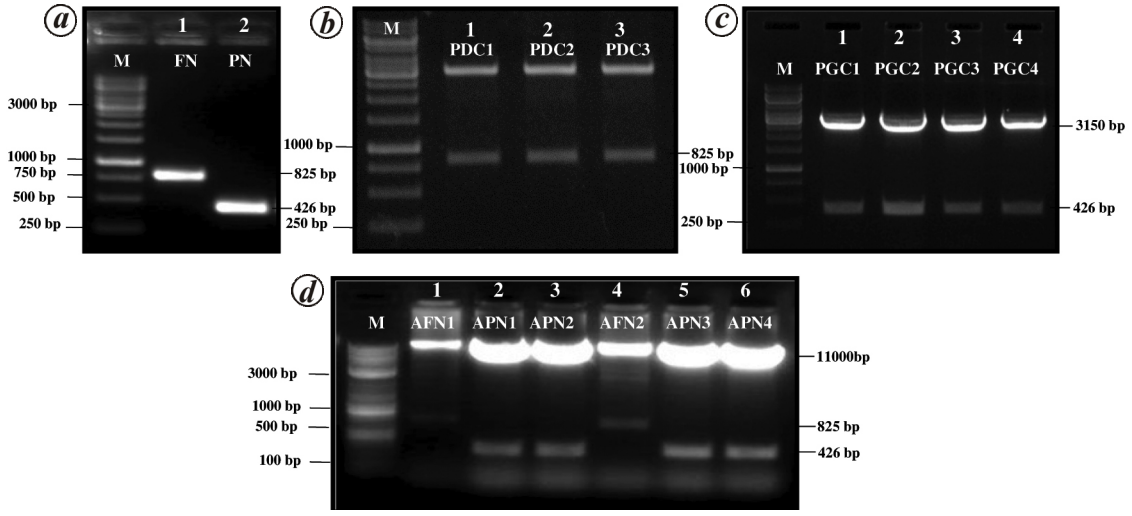

Figure 2. Development of *N* gene-based marker-free constructs using pCAMBIA 2300. ***a***, PCR amplification of 825 and 426 bp of *N* genes respectively; ***b***, Confirmation of recombinant clones by double restriction digestion using *Bam*HI and *Sac*I; ***c***, Confirmation of recombinant clones of pGEM-T Easy by *Xho*I; ***d***, Confirmation of recombinant clones of pCAMBIA 2300 harbouring *N* genes (825 bp: lanes 1 and 4) and (426 bp: lanes: 2, 3, 5 and 6) by restriction digestion.

Table 1. DAC-ELISA results of the agroinfiltrated watermelon using full and partial *N* gene constructs absorbance value (OD) at 405 nm

	Only agroinfiltration				Agroinfiltration + challenge inoculation					
					Full *N* gene construct		Partial *N* gene construct			
Genotype	Full *N* gene construct	Partial *N* gene construct	WBNV +ve	H	Local	Systemic	Local	Systemic	H	MI + CI
Asahi Yamato	0.14	0.11	0.40	0.11	0.46	0.12	0.43	0.17	0.17	1.03
Durgapura Lal	0.07	0.06	0.40	0.11	0.31	0.16	0.35	0.19	0.17	1.03
Sugar Baby	0.12	0.12	0.40	0.11	0.36	0.14	0.36	0.11	0.17	1.03
CD (0.01)	0.12	0.029	0.041	0.039	0.119	0.041	0.044	0.119	0.048	0.044
SEm±	0.034	0.008	0.012	0.011	0.034	0.012	0.012	0.034	0.014	0.012
CV	27.305	6.149	7.792	4.952	21.076	8.571	4.910	27.766	8.304	4.947

H, Healthy sample; +ve, Positive control; MI + CI, mock infiltrated and challenge inoculated.

confirmed the presence of 825 bp gene in pCAMBIA 2300 (Figure 2 *d*; lanes 1 and 4). *N* gene was then mobilized in *Agrobacterium* stain EHA 105, which was confirmed by PCR amplification using BM01F and BM02R primers.

Similarly, MF-pCAMBIA 2300 construct developed using partial *N* gene of 426 bp was amplified using specific primers (Figure 2 *a*; lane 2) and sub-cloned in pGEM-T easy vector and recombinants were confirmed (Figure 2 *c*). Colony PCR resulted in amplification of 426 bp product and restriction digestion of recombinant clones with *Xho*I showed release of 426 bp fragment of *N* gene along with inward pGEM-T easy (3150 bp) vector in 1% agarose gel electrophoresis (Figure 2 *c*). Eluted 426 bp product was cloned in already developed MF-pCAMBIA 2300. Colony PCR and restriction digestion (Figure 2 *d*; lanes 2, 3, 5 and 6) confirmed the presence of 426 bp gene in pCAMBIA 2300. The pCAMBIA 2300 harbouring 426 bp *N* gene was then mobilized in *Agrobacterium* strain EHA 105, which was confirmed by PCR amplification (data not shown).

Agroinfiltration was done in healthy leaves and then challenge-inoculated with WBNV (Figure 3). Similarly, 25–30 day-old healthy seedlings of Durgapura Lal were agroinfiltrated with the overnight grown culture of *A. tumefaciens* harbouring recombinant (MF-pCAMBIA 2300 N gene constructs) and non-recombinant (MF-pCAMBIA 2300 without any gene). Agroinfiltrated with full *N* gene constructs and challenge inoculated plants with WBNV from these three watermelon genotypes were tested by DAC-ELISA and OD values of 0.46, 0.31 and 0.36 were recorded in three replications from Asahi Yamato, Durgapura Lal and Sugar Baby respectively; whereas OD values of 0.43, 0.35 and 0.36 were recorded OD in the three watermelon genotypes respectively with agroinfiltrated and challenge inoculated with partial *N* gene construct (Table 1). After complete drying and necrosis of these agroinfiltrated and WBNV inoculated plants, the systemic young upper leaves remained healthy (Figure 3).

Results revealed negative reaction in DAC-ELISA in samples collected from the three genotypes (Asahi

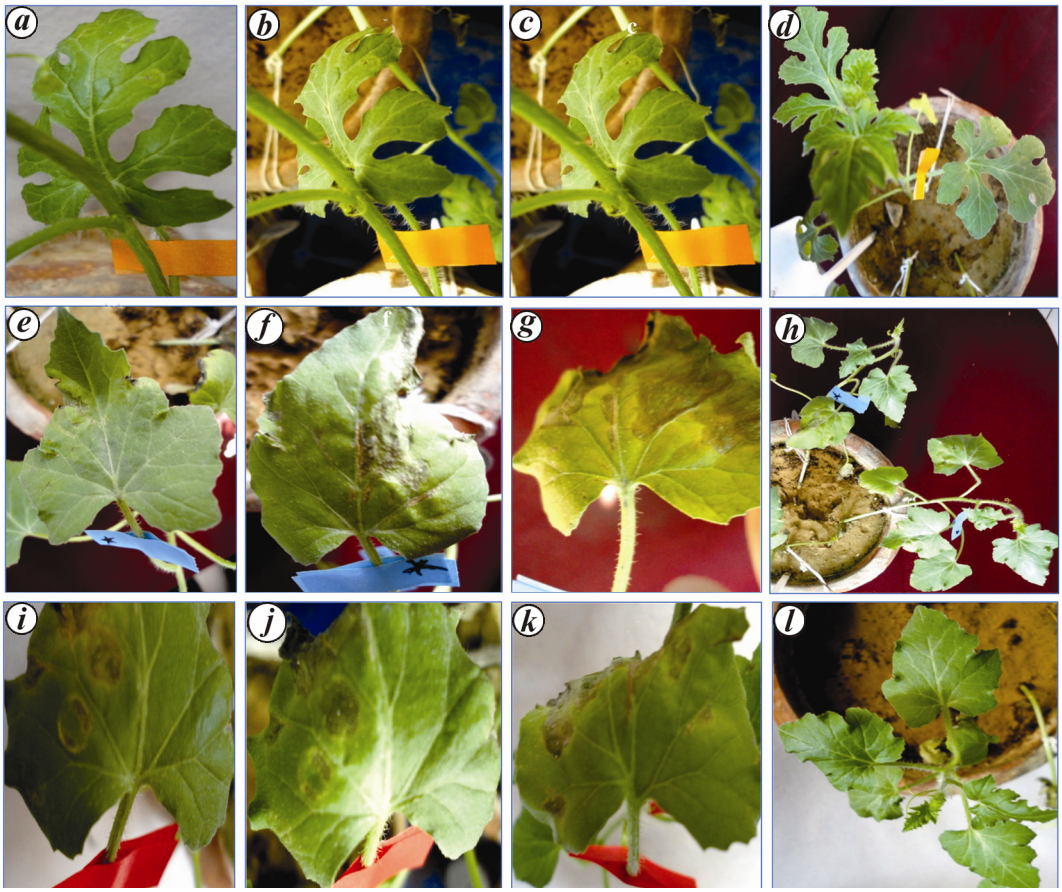

Figure 3. Agroinfiltration of full and partial *N* gene constructs in watermelon (*Citrullus lanatus* cv. Durgapura Lal) followed by challenge inoculation with WBNV (***a–d***); infiltration with non-recombinant *Agrobacterium* (***a***), 6 dpi (***b***) and 9 dpi's (***c***) and remained healthy (***d***). ***e***, partial *N* gene construct agroinfiltrated in watermelon showing necrotic margin at 3 dpai followed by necrosis of agroinfiltrated portion at 6 dpai; ***f***, severe necrosis of agroinfiltrated leaf at 9 dpai; ***g***, same plants of agroinfiltrated leaves dried and upper young leaves remained healthy (***h***); ***i***, Full *N* gene construct agroinfiltrated in watermelon showing necrotic spots at 3 dpai followed by necrosis of agroinfiltrated portion at 6 dpai (***j***), severe necrosis of agroinfiltrated leaf at 9 dpai (***k***), agroinfiltrated leaves dried and upper young leaves remained healthy (***l***).

Yamato: 0.14; Durgapura Lal; 0.07; Sugar Baby; 0.12) and 0.11, 0.05 and 0.12 in respective genotypes, agroinfiltrated with both the *N* gene constructs. The OD values from healthy and positive controls were 0.108 and 0.4 respectively. Young upper leaves were tested by DAC-ELISA from the same plants initially agroinfiltrated with full *N* gene construct and then challenge inoculated with WBNV. The OD values obtained were 0.12, 0.16 and 0.14 from Asahi Yamato, Durgapura Lal and Sugar Baby respectively. Similarly, OD values from young upper leaves of plants agro-infiltrated with partial *N* gene construct and then challenge-inoculated were recorded as 0.17, 0.19 and 0.11 for Asahi Yamato, Durgapura Lal and Sugar Baby respectively (Table 1).

The broad-spectrum resistance using inverted-repeat construct derived from *N* gene against several orthotospo virus species was developed[33]. Recently, *Nicotiana benthamiana* transgenic lines developed using WLm constructs containing conserved motifs of L-RNA of WSMoV showed resistance against tomato spotted wilt orthotospovirus (TSWV), groundnut yellow spot orthotospovirus (GYSV), impatiens necrotic spot orthotospovirus (INSV) and groundnut chlorotic fan-spot orthotospovirus (GCFSV)[24].

In India, transgenic resistance against GBNV in tomato, tobacco and peanut using full *N* gene in a sense or antisense orientations was developed[27–30]. Likewise, PTGS *N* gene constructs of 355 bp of GBNV in ihp and SHUTR vector were developed against GBNV in groundnut[27,28]. Little information was available for *N* gene constructs conferring resistance to WBNV in watermelon. Hence, the present study was designed to develop the truncated (426 bp) and full (825 bp) *N* gene constructs for developing resistant watermelon against WBNV.

Marker-free constructs were developed without the use of *npt*II as a selection marker as marker-free transgenic plants delay symptom development[34]. Marker-free transgenic plants resistant to ageratum yellow vein virus (AYVV) and melon yellow spot orthotospovirus (MYSV) were developed and this provided a novel way to control

both DNA and RNA viruses to address bio-safety concerns[34]. Both constructs developed in this study were marker-free.

Scanty information was available with agroinfiltration in watermelon using *N* gene constructs. Watermelon was the most suitable plant for agroinfiltration because it formed clear water soaked lesion in agroinfiltrated portions of the leaves. Optimization of genetic transformation of watermelon using marker-free constructs is required and needs to be established to develop transgenic watermelon resistance against WBNV in India.

In this study, full and partial *N* gene marker-free constructs (pCAMBIA 2300) were developed for utilization in the development of a putative transgenic against WBNV in watermelon. The study validated these constructs in watermelon through agroinfiltration. *N* gene conferred resistance to WBNV in watermelon through hypersensitive reaction (HR). Watermelon is a suitable host for transient expression studies. This is the preliminary confirmation of the successful agroinfiltration of *N* gene constructs in watermelon. The agroinfiltrated portion turned into necrotic lesions within 3–6 dpi due to HR reaction; interestingly the young upper systemic leaves remained free from the virus till maturity. This is the way forward for developing WBNV in watermelon in India.

Conflicts of interest: The authors declare that there is no conflict of interest.

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Dual transmitter–receiver electromagnetic system for lateral boundary detection of subsurface formations

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A new frequency domain electromagnetic system, based on different working principle has been designed and its efficacy tested over the existing systems through laboratory-scale-model studies. In this system, two transmitter coils have been employed to generate a magnetic null plane at their geometric centre. The receiver coil is placed in the null plane to record the induced secondary field. The interaction of the primary field is almost negligible on the secondary field recorded by the receiver. We present the theory and physical model results describing the system parameters and efficacy. The testing through physical model studies suggests an increased depth of detection in this new configuration compared to the existing systems. In terms of secondary field, the strength of the anomaly reflects the magnetic permeability/susceptibility difference of the subsurface medium on either side of the receiver. The study concludes that there is significant increase in depth of investigation and secondary field strength in this system over the existing conventional frequency domain systems and also more robust for boundary detection.

Keywords: Conducting bodies, electromagnetic system, magnetic permeability, physical model studies, susceptibility.

ELECTROMAGNETIC (EM) methods are popular for a wide variety of applications in exploring the internal structure of objects by transmitting the primary electromagnetic field and analysing the induced secondary field intensities. Similar mechanism is involved in the geophysical electromagnetic exploration for measuring conductivity/susceptibility of the subsurface formations to map the mineral/ore deposits[1]. Geometrical electromagnetic sounding is a popular technique, in which the separation between the transmitter and receiver coils is increased to achieve deeper penetration[2]. The skin depth is the most important and interesting phenomenon that restricts the penetration of the fixed frequency electromagnetic energy up to a constant depth for a fixed transmitter (Tx) and receiver (Rx) separation within a subsurface layer with specific electrical conductivity[3,4]. Most of the frequency domain electromagnetic systems utilize the concept of skin depth to scan the subsurface by decreasing the frequency of the primary field. Such frequency sounding is the most popular method since the past five decades in geophysical electromagnetic exploration[5]. These systems are handy in near subsurface study, such as groundwater exploration in hard-rock terrains, soil pollution study, mineral exploration, etc.

Different techniques and coil configurations have been developed to nullify the influence of strong primary field on the receiver in frequency domain EM exploration. Recently, researchers have designed the vertical primary decoupled coil configuration (VPDCc) to nullify the effect of primary field on the receiver[6,7]. They have suggested placing the receiver coil at a strategic position where the primary magnetic field horizontal component is zero. The performance and stability of VPDCc have been tested and found efficient among the existing conventional coil configurations using laboratory studies. However, the complicated non-coplanar and non-coaxial geometrical set-up of the transmitter and receiver coils in the VPDCc reduces its wider applicability.

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