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Synthesis and Screening of Anilates as Chemical Hybridising Agents for Wheat (*Triticum aestivum* L.)

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ABSTRACT. *Hybrid wheat technology has the potential in enhancing the productivity levels by about 20–25%. Chemical hybridising agents (CHAs) are useful tools in heterosis breeding of crops facilitating "two-line" approach. Fourteen anilates were synthesised and screened on 2 genotypes of wheat (PBW 343 and HD 2687) at premeiotic stage and at 2 different concentrations (1000 and 2000 ppm) to understand the structure activity relationships as CHAs. The field trial was conducted following Randomised Block Design (RBD) with 3 replicates. Ethyl 4'-fluoro oxanilate appeared to be the best in inducing 93.75% male sterility in HD 2687 and 95.37% in PBW 343 at 2000 ppm concentration. Ethyl 4'-fluoro malonanilate induced 78.13 and 83.15% male sterility in HD 2687 and PBW 343 respectively at 2000 ppm concentration. Ethyl 2'-methoxy and ethyl 4'-chloro oxanilates exhibited moderate activity as potential CHAs. Para substituted anilates had shown better CHA-activity than ortho-analogues and the least by meta. Side chain elongation by a methylene bridge significantly lowered the activity. The most effective fluore-anilates did not show any adverse effect on different growth and yield parameters including female fertility, and thus have a great potential as CHAs for wheat.*

INTRODUCTION

The Asia-Pacific region has less than 30% of the global agricultural land however, sustains more than half (3 billion) of the world's population. The almost static area under wheat during the last one decade illustrates that expansionist agriculture is no longer possible in Asia. The only obvious option left is to have vertical intensification, which can be possible through an increased productivity using heterosis breeding. Exploitation of heterosis at commercial level depends on the availability of either cytoplasmic-genetic male sterility (CGMS) or through certain chemicals called chemical hybridising agents (CHAs). CHAs are chemicals that aid in the production of hybrids and the expression embraces all such terms as male sterilant, selective male sterilant, pollen suppressant, pollenocide, androcide, *etc*.. A cogent argument in favour of this broad-base generic terminology has been advanced (McRae, 1985). The success of hybrid wheat programme other than CHA approach lies on 2 key elements *viz*., development of stable CGMS lines and perfect restorer system in three-line approach. However, this methodology is off-set by many obstacles and limitations such as non-availability of breeding stocks containing CGMS and restorer system, their instability and the labourious method of heterosis breeding. Besides being tedious and time-consuming, this technique becomes sometimes untenable due to the lack of a consistent restorer system for genetic restoration of fertility (Guilford *et al*.,

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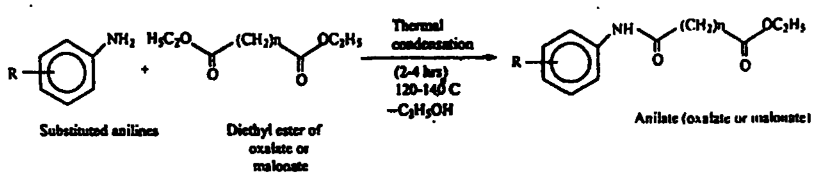
1992). The alternative approach using CHAs neither requires restoration nor conversion of parental lines to a CGMS background. CHA facilitates cross breeding in plant species with perfect flowers by selectively sterilising male sex cells or by interrupting microsporogenesis to prevent self- pollination and to promote fertilisation by an outside pollen source thus offering opportunities to develop hybrids. The technology for making hybrids with a CHA is, in essence, identical to the CGMS method. The only difference is that the functional male sterility in the female parent is induced with a chemical rather than through genetic manipulation (Tu and Banga, 1998). France, Italy, USA and Australia are already ahead in commercialising wheat hybrids using CHA approach. Considerable research effort for hybrid wheat development in South-East Asian region is yet to bear fruit. The search for gametocides in the fifties and sixties was rather random (McRae, 1985) and from the chemicals already used in agriculture such as plant growth regulators like maleic hydrazide, ethrel, abscisic acid and herbicides like 2, 4-D, dalapon which expectedly have strong phytotoxic response. Thus chemicals with targeted action are being now searched all over the world. Anilates as chemical hybridising agents have been successful in rice (Ali *et al.*, 1999a). The present study was, therefore, undertaken to synthesise and screen 10 oxanilates and 4 malonanilates in 2 different concentrations (1000 and 2000 ppm) on 2 genotypes of wheat (PBW 343 and HD 2687) at premeiotic stage and to understand the underlying structure-activity relationships in anilates as CHA.

MATERIALS AND METHODS

Synthesis of anilates

Oxanilates and malonanilates were prepared by condensation reaction of substituted anilines with corresponding diethyl esters as given in Scheme 1. Analar grade anilines obtained from ALDRICH Chemicals, USA were used.



Scheme 1 : Synthesis of anilates

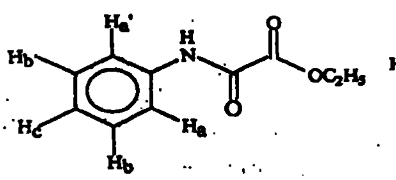
Anilates	R
Oxanilate (n=0)	4'-F, 4'-Cl, 3'-Cl, 2'-Cl, 4'-Br, 3'-OMe, 2'-OMe, 2'-NO_2, 3'-NO_2 and 3'-CH_3
Malonanilate (n=1)	4'-F, 3'-OMe, 2'-OMe and 2'-NO_2

General methods of preparation and purification of anilates

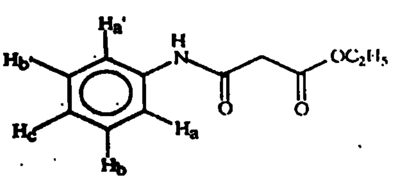
Diethyl ester of oxalate or malonate (0.03 mol) and different substituted anilines (0.025 mol) were refluxed together for 2–4 h in an oil bath. The coarse of reaction in each case was followed by thin layer chromatography (TLC) or gas chromatography (GC). The dark coloured aliquot so produced was cooled to below 90°C to let the product to solidify. The solid thus formed was triturated with boiling ethanol and the hot solution was filtered. The required anilates were obtained as crystalline solids by crystallisation. The purity of the crystalline product was confirmed by melting point, TLC, GC and HPLC as deemed necessary. The yields were generally over 80%.

The structures of anilates were confirmed by different spectroscopic techniques. The Proton Magnetic Resonance (^1H-NMR) spectra were recorded on a Varian EM 360L, 60 MHz instrument in $CDCl_3$, using tetramethylsilane (TMS) as an internal standard. The infra red (IR) spectra of compounds as KBr pellets were recorded on a Nicolet Fourier Transform Infra Red spectrometer (Model Impact–400). The mass spectra were recorded on a FISONS GC-MS in which GC-8000 series was coupled with EI-mass detector (MD–800). The ^1H-NMR spectral data of the anilates are summarised in Table 1.

Table 1. Chemical shifts (δ) of different protons in anilates.



Oxanilate (1 - 10)



Malonanilate (11 - 14)

C. No. R[(a)]	δ (ppm) -CH_3	-OCH_2	Ha	H'a	Hb	H'b	Hc	-NH-	-CH_2-CO	Ar-OCH_3
1. 4'-F	1.65	4.70	7.95	8.10	7.40	7.40	-	9.44	-	-
2. 4'-Cl	1.52	4.60	-	7.95	7.65	7.65	-	9.36	-	-
3. 2'-Cl	1.40	4.45	8.65	-	7.25	7.25	7.25	9.60	-	-
4. 3'-Cl	1.50	4.50	7.55	7.75	7.35	-	7.30	9.10	-	-
5. 4'-Br	1.45	4.45	7.65	7.65	7.65	7.65	-	9.12	-	-
6. 2'-OMe	1.43	4.32	-	8.25	6.85	6.85	6.85	9.23	-	3.88
7. 3'-OMe	1.45	4.60	7.60	7.55	-	7.48	7.00	9.22	-	3.91
8. 2'-NO_2	1.58	4.60	-	9.11	8.55	7.90	7.50	10.36	-	-
9. 3'-NO_2	1.30	4.30	8.40	7.95	-	7.50	7.50	9.10	-	-
10. 3'-CH_3[(b)]	1.38	4.28	7.30	7.30	-	7.10	6.80	9.38	-	-
11. 4'-F	1.23	4.15	7.64	7.64	7.11	7.11	-	9.70	3.46	-
12. 2'-OMe	1.21	4.00	-	8.34	6.95	7.07	7.01	9.52	3.56	3.89
13. 3'-OMe	0.75	3.75	7.50	6.90	-	6.55	6.40	8.55	3.15	3.23
14. 2'-NO_2	1.50	4.55	-	9.10	8.50	7.80	7.40	10.30	4.20	-

(a) Substituent in the aromatic ring (b) δ for Ar-CH_3 appeared at 2.25 C. No.: Compound number

Field trials

Two high yielding cultivars of bread wheat (*Triticum aestivum* L.) namely, PBW 343 and HD 2687 released for Cultivation in North Western Plain Zone (NWPZ) comprising Punjab, Haryana, Western Uttar Pradesh and parts of Rajasthan in India and having high yield potential and high rust resistance were chosen for chemical induction of male sterility. The experiment was laid out in a Randomised Block Design (RBD) with three replicates. Fourteen chemicals (10 oxanilates and 4 malonanilates) at two levels of concentrations (1000 and 2000 ppm) with one control were applied on PBW 343. In all, there were $14 \times 3 \times 2 + 1 = 85$ sub-plot treatment combinations. A similar experiment was laid out for cv. HD 2687. Seeds of both of the wheat varieties *i.e.*, PBW 343 and HD 2687 were sown using a seed drill in November, 1998, using 100 kg N, 60 kg P_2O_5 and 40 kg K_2O following a 100 kg ha^{-1} seed rate at the Indian Agricultural Research Institute (IARI), New Delhi. Row to row distance was kept at 23 cm. Four rows of 2 m length were taken as a plot. Other optimum agronomic practices were also followed.

The test compounds were sprayed at premeiotic stage when the length of the spike emerging out of the 1st node was about 7–8 mm (Zadoks *et al.*, 1974). This occurred 60 days after sowing (60 DAS). Emulsifiable concentrates (EC 17) of the anilates were prepared in solvent chlorobenzene using polyoxyethylene sorbitan monooleate (F.W. ~ 1200) (Tween-80®) as emulsifier. To ensure phytosafety of chlorobenzene, a blank solvent emulsion without anilates was sprayed on the crop well in advance. No serious phytotoxic symptoms were visible 2–3 days after spray. Spray emulsions (1000 and 2000 ppm) were obtained by appropriate serial dilution of EC 17 with tap water. The formulations were sprayed on the crop to run off on 3 replicate plots of 1 m length of 2 lines containing about 200 tillers, keeping the outermost 2 lines as pollinator lines. Five spikes of each treated plots were covered with rainproof paper bags at pre-emergence stage (Zadoks *et al.*, 1974) when awns were just emerging. Pollen sterility and seed set were chosen as the parameters of efficacy of the CHAs. For study of pollen sterility, anthers from 3–4 florets were smeared together over a drop of acetocarmine (1%) and examined under a light microscope. Five each of bagged and unbagged spikes including one control were harvested at maturity. To study the floret sterility, number of fertile (filled) and sterile (unfilled) grains were counted and per cent male sterility was computed. Similarly, data on parameters such as plant height, spike length, spikelet number and thousand grain weight were recorded on 12 plants of each treatment and untreated controls. Germination test was conducted by placing the water-soaked blotting paper containing seeds in the petridishes for 5 days. The data were subjected to analysis of variance (ANOVA) of factorial RBD.

RESULTS AND DISCUSSION

Characterisation of chemical structures

The ^{1}H-NMR spectra of anilates contained a triplet at δ 1.47 ± 0.10 ppm [$-CH_3$] in oxanilates and at δ 1.17 ± 0.27 ppm in malonanilates. A quartet centred at δ 4.48 ± 0.15 ppm [$-OCH_2$] in oxanilates and at δ 4.11 ±.0.29 ppm in malonanilates, a broad singlet at δ 9.39 ± 0.38 ppm in oxanilates and at δ 9.52 ± 0.63 ppm in malonanilates corresponding to -NH proton. The labile $-CH_2-$ protons in malonanilates appeared at δ 3.59 ± 0.38 ppm. In the mass spectra of oxanilates, the base peak was found to be either the M^+ ion peak or protonated aryl isocyanate moiety (M-73), whereas in malonanilates, anilines (M-114) appeared to be the base peak. IR-spectra showed characteristic peaks for symmetrical-NH stretching vibrations at 3339.3 ± 16.0 cm^{-1} for oxanilates and 3330.2 ± 42.5 cm^{-1} for malonanilates. Relative efficacy of anilates for selective induction of male sterility (ms) was studied on 2 genotypes (HD 2687 and PBW 343) at 2 concentrations (1000 and 2000 ppm) sprayed at premeiotic stage.

Effect of oxanilates on induction of male sterility (ms)

Maximum ms was induced by ethyl 4'-fluoro oxanilate which differed significantly (P=0.05) over other oxanilates in the series in the varieties studied (Table 2). It was closely followed by ethyl 2'-methoxy oxanilate. Ethyl 4'-chloro oxanilate, ethyl 2'-nitro oxanilate and ethyl 2'-chloro oxanilate induced moderate ms ranging from 60–40% at 2000 ppm concentration, but they induced significantly low ms at 1000 ppm concentration (Table 2). All other oxanilates were found to record very low (≤30%) ms induction on the varieties studied.

Table 2. Relative per cent male sterility induced by different oxanilate formulations on HD 2687 and PBW 343.

Oxanilates	Male sterility (%) # in genotypes			
	HD 2687		PBW 343	
	1000 ppm	2000 ppm	1000 ppm	2000 ppm
4'-Fluoro	79.79	93.75	88.70	95.37
4'-Chloro	20.49	61.04	41.48	68.33
3'-Chloro	8.12	13.13	10.56	15.55
2'-Chloro	9.79	39.99	20.18	52.59
4'-Bromo	8.20	20.42	6.30	12.78
2'-Methoxy	36.25	59.79	53.52	70.00
3'-Methoxy	8.33	19.31	10.55	25.74
2'-Nitro	24.37	44.17	32.78	52.22
3'-Nitro	4.58	8.13	12.03	16.67
3'-Methyl	0.83	8.13	1.85	4.63
LSD (P=0.05)	0.87		1.44	

Mean of three replicates

Earlier studies with variety Pusa 150 of rice indicated almost similar results in which ethyl 4'-fluoro oxanilate was found to induce 100% pollen sterility, closely followed by ethyl 2'-nitro oxanilate and ethyl 2', 5'-dimethoxy oxanitate inducing 92–97% male sterility (Ali *et al.*, 1999a).

Effect of malonanilates on induction of ms

Maximum ms was induced by ethyl 4'-fluoro malonanitate, which differed significantly (P=0.05) over other malonanitates on the varieties studied (Table 3). Others had shown very low level of ms induction in both the concentrations (1000 and 2000 ppm) tested.

Table 3. Relative per cent male sterility induced by different malonanilate formulations on HD 2687 and PBW 343.

Malonanilates	Male sterility (%) # in genotypes			
	HD 2687		PBW 343	
	1000 ppm	2000 ppm	1000 ppm	2000 ppm
4'-Fluoro	66.25	78.13	77.22	83.15
2'-Methoxy	7.71	18.13	10.00	17.04
3'-Methoxy	4.17	18.13	7.78	17.96
2'-Nitro	0.63	7.15	1.36	2.41
LSD (P=0.05)	0.51		1.94	

Mean of three replicates

The most effective anilates helped in the opening of glumes in tile cleistogamous wheat florets increasing the chance of cross pollination from an outside pollen source, without much manual effort (Plate 1).

Pollen sterility stain test.

From the acetocarmine stain test it was seen that the sterile grains were transparent thereby confirming the disintegration of cytoplasm and nucleus in the sterile pollen (Plate 2). In contrast, fertile pollen from control plots stained uniform deep red colour, confirming the induction of ms in various treatments. The data of per cent ms obtained from field under bagged test strongly correlated with those of per cent pollen sterility as measured by acetocarmine stain test. The 'r' values were very high at 0.9988 ± 0.001 ranging from 0.9977–0.9999.

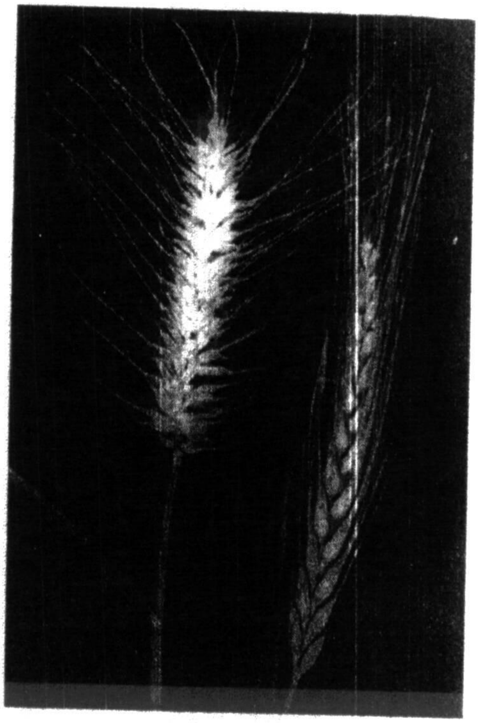


Plate 1. **Opening glumes of treated plants (left)** as compared to closed glumes of untreated plants (right).

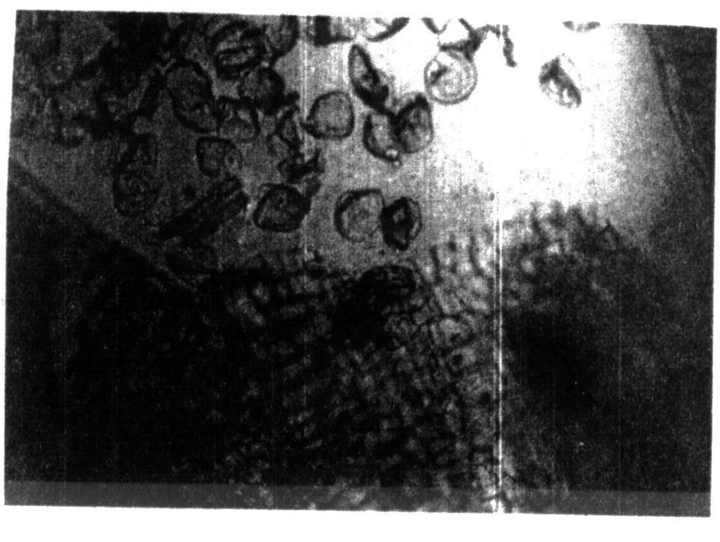


Plate 2. **Sterile pollens of wheat treated by ethyl** 4'-fluoro oxonilate.

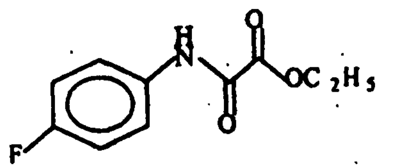
Structure activity relationship

The *para*-substituted anilates appeared to be better than *ortho*-analogues of the same substituents, the least effective being *meta*-substituted analogues in both wheat genotypes. Between the pairs of oxanilates, the order was 2'-OCH_3 > 3'-OCH_3 and 2'-NO_2 > 3'-NO_2. A comparison of 3 *ortho*-substituted oxanilates reveals that the order of induction of ms in both the genotypes over 2 concentrations decreased thus : OCH_3 > Cl > NO_2. Currently, we are evaluating 4'-methoxy and 2'-fluoro oxanilates in the light of present findings. A comparison of halogen substituted oxanilate at *para*-position shows a drastic reduction in the induction of ms from F through Cl to Br. This may mean that the activity may be directly correlated with electronegativity and inversely correlated with steric factor. A consideration of inductive but not resonance effect mig' partially explain the activity-ranking of oxanilates (*i.e.*, 4'-F > 2'-OCH_3 > 4'-Cl > 2'-NO_2) in which the 4 substitutents all have shown negative inductive (-I) effect. In addition, the steric effect seems to be operating for the higher activity of *para* and not *ortho*-substituent. A comparision of induction of ms by 2 fluoro analogues (1 and 11) on both the varieties at 2 test concentrations (Table 2 and 3) reveals that 1 was superior to 11 in performance. Chemically, a methylene (-CH_2-) bridge distinguishes 11 from 1. Thus, elongation of the side chain in 1 by introducing a methylene bridge as in 11 has caused reduction in ms induction. Also, ethyl 2'-methoxy oxanilate is more active as compared to ethyl 2'-methoxy malonanilate. Earlier results with variety Pusa-150 of rice indicated similar trends governing the structure-activity relationship (Ali *et al.*, 1999b).

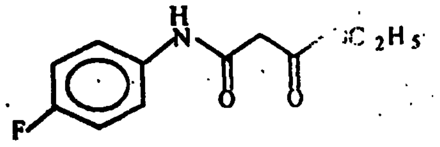
Examination of possible side effects

The anilates were further examined for possible side effects which could be counter productive. Both the fluoro analogues of oxanilate and malonanitate did not show any significant adverse effect on plant growth, selectivity in male sterility and yield parameters. There was a marginal reduction in the plant height of the plants treated with 1 and 11 on both the varieties in comparison to emulsion control treatment (Table 4). Spike length was not significantly reduced from the control values in the varieties studied (Table 4). In the most effective fluoro and methoxy oxanilate treatments, the reduction in spikelet number is very meagre in comparison to the control at 2000 ppm test concentration (Table 4). For fluoro maloanilate the reduction is about 0.07 cm as compared to control. The thousand grain weight of HD 2687 for fluoro and methoxy anitates at 2000 ppm test concentration were not much different from the control. The trend was identical in genotype PBW 343. Regarding germination test, fluoro and methoxy anitates gave more than 90% seed germination irrespective of the dose of application and genotype. The data on per cent female fertility as shown under Table 4 clearly revealed that the chemicals 1 and 11 did not affect this phenomenon implying thereby that these chemicals are selective in the induction of male sterility. The key to the use of a CHA in the commercial production of hybrid wheat is to achieve high male sterility while maintaining a high degree of female fertility. The most promising 4'-fluoro and 2'-methoxy oxanilates recorded very high per cent female fertility in both the genotypes tested (Table 4). The comparison of published data (Mahajan *et al.*, 2000) in the performance of CH 9832 on PBW 343 with our best CHA points to the superiority of the latter in containing adverse effects. The vital statistics of performance of ethyl 4'-fluoro oxanilate, ethyl 4'-fluoro malonanilate on PBW 343 and HD 2687 given in Table 4, clearly prove their worthiness as promising CHAs.

Table 4. Performance indicators of fluoro analogues on 3 cultivars of wheat sprayed at 7–8 mm spike length stage at 2000 ppm test concetration.



Ethyl 4'-fluoro oxanilate(I)



Ethyl 4'-fluoro malonanilate (II)

Compound No.	Variety	Plant growth parameters						
		Plant Height (cm)	Spike Length (cm)	Spikelet No.	Seed set in Un-bagged Condition	Test Weight (g)	Germination (%)	Female Fertility (%)
I	HD 2687	78.83	10.66	15.90	53.65	36.66	95.00	99.05
	PBW 343	76.37	10.63	18.23	53.12	37.64	95.00	99.88
II	HD 2687	78.20	10.63	15.90	53.41	36.67	97.00	98.63
	PBW 343	76.33	10.60	18.13	52.81	37.00	96.85	98.13
Emulsion control	HD 2687	79.62	10.86	16.07	54.16	36.98	99.23	100.00
	PBW 343	78.13	10.79	18.30	53.18	37.92	98.62	100.00
LSD (P=0.05)		0.33	0.10	0.09	0.32	0.32	0.06	1.13

CONCLUSIONS

Ethyl 4'-fluoro oxanilate followed by ethyl 4'-fluoro malonanilate have shown very high and selective induction of male sterility. More significantly they have not shown any serious adverse efect on different plant growth and yield parameters. Ethyl 4'-fluoro oxanilate can serve as the CHA for the identification of useful heterotic combinations in wheat. Simultaneously, efforts should be made to identify better and more potent analogues as well as better formulations.

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