Effect of Five Pharmaceutical Substances contained in Urine on the Germination of Cress and Cereal Seedlings

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Abstract Usage of urine in agriculture includes the risk of spreading pharmaceutical residues on to agricultural fields. It is unclear if concentrations applied can cause any adverse effects during germination. Therefore, germination tests of cress and four different cereals were performed where the seeds were germinated in urine-water mix containing one up to five different pharmaceutical substances in raising concentration. The seedlings show sensitivity against pharmaceutical agents but not at the concentration levels the agents are expected for average German urine. Aside, the urine matrix itself is much more affecting the seedlings due to its specific matrix than the active agents. Also, in certain cases reactions of seedlings towards the pharmaceutical substances can be observed. Overall, it can be concluded that the potential effect of pharmaceutical substances contained in urine towards plants cannot be determined in germination experiments.
Keywords Pharmaceuticals; urine; germination; cress; cereals

INTRODUCTION

Urine is discussed as an alternative fertilizer for agriculture as it contains relatively high concentrations of the macronutrients nitrogen, phosphorus, and potassium (Vinnerås and Jönsson, 2002; Simon and Clemens, 2005; Ganrot et al., 2007; Muskolus, 2008). But this usage of urine includes the risk of spreading pharmaceutical residues on to agricultural fields (Winker et al., 2008). Little is known on the effects of pharmaceutical substances they exaggerate on plant physiology and development. These aspects are of major interest especially for agricultural crops with regards to fertilization with urine.

It is known from literature that pharmaceuticals can affect plant development when dosed in sufficient concentrations (Winker, 2009) in a positive, enhancement of plant development (Goodman, 1959; Kopcewicz, 1969), as well as negative way (von Euler and Stein, 1955; Goodman, 1959). Nevertheless, nearly no investigations focused on application of pharmaceuticals by urine except Schneider (2005) and Winker et al. (2009). In this research the focus laid on uptake of certain pharmaceuticals by rye grass. As the setup did not allow to

apply too many different pharmaceuticals: Schneider (2005) applied diclofenac (DIC), sulfamethoxazole (SMX), or sulfamethazine but in concentrations $5*10^5$ (DIC; DIC concentration in AGU is 11.4 $\mu g\ l^{-1}$) and $9*10^5$ (SMX; SMX concentration in AGU is 311 $\mu g\ l^{-1}$) higher than expected for an average German urine (AGU, Winker et al., 2008) while sulfamethazine is not even present in AGU at all; and Winker et al. (2009) applied carbamazepine (CZ), ibuprofen (IBU), and 17α-ethinylestradiol (EE2) alone and in combinations in the expected as well as higher dosed concentrations of those in AGU. Therefore, this study wants to use germination tests which are less time and space consuming to investigate the effects of pharmaceutical substances contained in urine with more pharmaceuticals on a wider range of plant types in the sensitive phase of germination.

The question stated was whether concentrations applied by urine fertilization are causing any adverse effects during germination and how these effects would manifest themselves. Especially during germination, plants show a high sensitivity to environmental conditions.

MATERIAL AND METHODS

Setup. Cotton pads were laid into petri dishes and 20 seeds were placed into each dish. Each treatment had four repetitions. Every experiment was accompanied by a control of 8 jars receiving a urine-water mix without pharmaceuticals and was run for 10 days.

Pharmaceuticals. The selected pharmaceuticals were the anti-epileptics carbamazepine (CZ, CAS-N^o. 298-46-4) and primidone (PI, CAS-N^o 125-33-7), the antiphlogistic ibuprofen (IBU, CAS-N^o. 15687-27-1), and the sex hormones 17α-ethinylestradiol (EE2, CAS-N^o. 57-63-6) and 17ß-estradiol (E2, CAS-N^o. 50-28-2). All pharmaceuticals were purchased from Sigma-Aldrich: IBU, minimum 98 % GC; EE2, minimum 96 % HPLC; E2, 97 %; for CZ and PI was no information on purity was provided. Concentrations of the substances were adjusted towards the urine fraction in the urine-water mix (UW mix) according their expected concentrations in AGU (Winker et al., 2008). The concentrations raised in multiples of 10 (for further details see Table 1).

Table 1: Exemplary demonstration how the applied concentrations were determined in the urine fraction of the UW mix and their respective abbreviations. The table shows the procedure for the example of the 1000 fold concentration related to the expected concentration of the pharmaceutical agents in average German urine (AGU).

Active agent	Abbre-viation	Expected concentration in AGU (mg l^{-1}) [1]	Higher dosing: 1000 fold of AGU (mg l^{-1})
Carbamazepine	CZ	0.058	58
Primidone	PI	0.086	86
Ibuprofen	IBU	0.80	800
17α-Ethinylestradiol	EE2	0.000025	0.025
17ß-Estradiol	E2	0.0053	5.3

[1] according to Winker et al. (2008)

Urine-water mix (UW mix). 40 ml of liquid were applied to each jar. As urine itself delays germination (Simon and Clemens, 2005), a UW mix was used: 1 part urine and 20 parts of water. The urine originated always from the same male and was collected in the 48 hours prior the experiment's start. Male urine was used to avoid the fluctuation of hormones occurring in urine of females due to their menstrual cycle.

Seeds. Seeds of cress as well as various cereals were used. The cereals were winter wheat (Hermann of Limagrain GmbH), winter barley (Campanile of Limagrain GmbH), oat (Aragon, Saaten-Union GmbH), and winter rye (Boresto, BayWa AG). Cress was tested for single substances as well as for combinations of 2 up to 5 substances, cereals only for single substances.

Evaluation. The germination was determined by counting the seedlings according their development. Cress seedlings were categorized as "not germinated", "germinated without roots", and "fully germinated". Cereal seedlings were classified according the BBCH-scale (Meier, 2001; BBCH stands for the three institutions involved: Biological Federal Institution for Agriculture and Forestry, Federal Plant Variety Office and the chemical industry.). Additionally, the dry weight of the germinated seedlings was determined. The jars were posed in the drying cabinet for 100 h at 60°C. Then the weight difference (empty jar plus cotton pad vs. dried jar with pad and seedlings) was determined. The weight added by the UW mix was not considered in this method, although some minerals remained in the jar after drying.

Statistics. Outliers were eliminated with the Grubbs-test for outliers. Then, results were statistically evaluated with SPSS 15. A one-way ANOVA was accomplished as a one-way descriptive method in cooperation with a Student-Newman-Keuls procedure. α was set to be 0.05 to determine a significant difference between various treatments.
The limiting concentration was determined as that concentration resulting in the impact (which was statistically determined) towards the seedlings in comparison to the lower dosed treatments not showing effects in a positive nor negative way.

RESULTS AND DISCUSSION

Seedlings showed visual differences during germination and in the end of the testing period. Nevertheless, in most cases a statistically proofed reaction was not determined by using ANOVA (Table 2).

Table 2: Results of the germination experiments for the addition of one active agent. > indicates that the limiting concentration was not reached and lies above the tested level.

Substance*	Cress	Winter wheat	Winter rye**	Winter barley	Oat
EE2	>1.000.000 fold	> 1000 fold	>1000 fold	>1000 fold	> 1000 fold
E2	>10.000 fold	> 1000 fold	>1000 fold	> 1000 fold	> 1000 fold
CZ	>10.000 fold	> 1000 fold	AGU conc.	>1000 fold	> 1000 fold
PI	10 fold - better	> 1000 fold	1000 fold - worse	>1000 fold	> 1000 fold
IBU	>1000 fold	> 1000 fold	1000 fold - better	> 1000 fold	> 1000 fold

* The full names of the substances are provided in Table 1.

** "worse": the concentration let to a negative effect of the dry weight; "better": the concentration let to a statistically relevant increase of the dry weight.

In most cases when the pharmaceutical agent was added on a seed type neither a delay nor an enhancement was statistically determined. Aside the statistical evaluation some tendencies for certain seed / agent pairs were found:

- E2 and CZ showed higher weights for oat with raising concentrations,
- while EE2 showed a lower weight of oat seedlings for all treatments compared to the control.
- For rye the weight decreased continuously with raising concentrations of EE2,
- the same effect was observed in the case of wheat when EE2 and E2 were applied.

Aside, a visual effect could be observed in the case of ibuprofen (Figure 1). The roots of all cereals tried to avoid the contact with the UW mix containing ibuprofen. This was shown best for rye. This observation was not reflected in the statistical analysis.

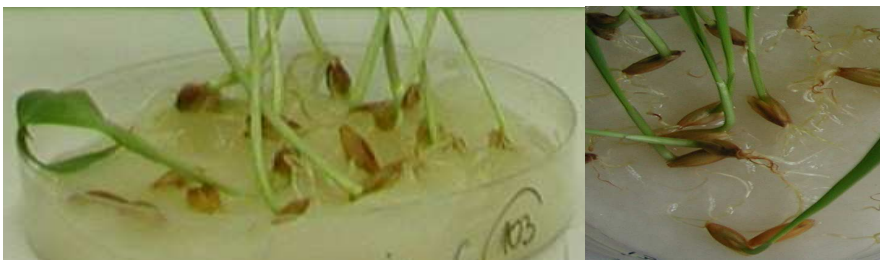

Figure 1: Winter barley roots trying to avoid the contact with the liquid and oat roots getting brown and curly. The UW mix contains the 1000fold dosing (compared to an expected concentration in AGU) of ibuprofen in its urine part.

The treatments which contained combinations of 2 up to 5 active agents were only done with cress due to the results contained with cress before cereal experiments were started as well as space and time constraints. The combinations did not show clear tendencies (Figure 2).

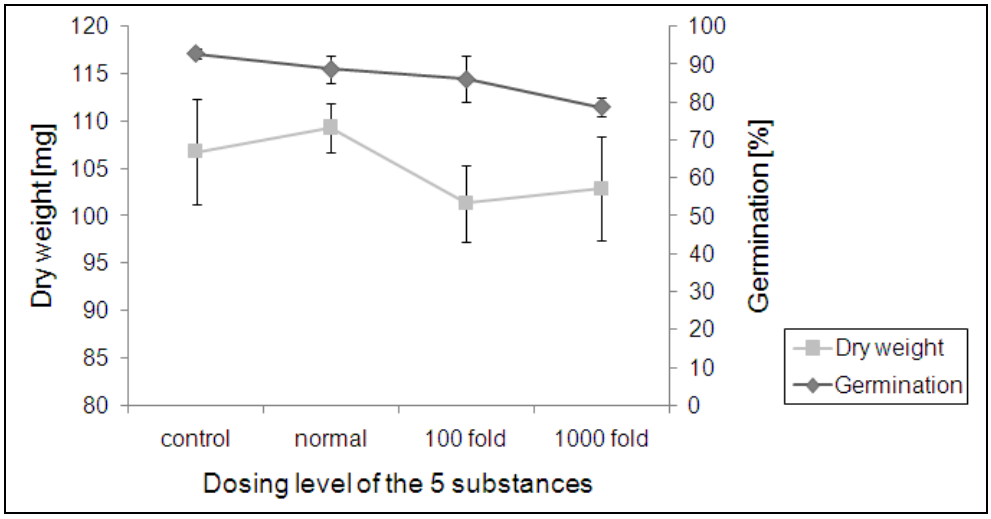

Figure 2: Change of dry weight and germination (measured by the seedlings germinated with roots) for cress exposed to a combination of all 5 active agents at different levels. Error bars show the standard deviation from the average among the 4 replications.

Also, the visual effect of ibuprofen was not observed anymore (Table 3). Jars exposed to UW mixes containing ibuprofen did not behave worse than others regarding the amount seedlings germinating and the dry weight. Aside it has to be mentioned that in the case of combinations of three substances or more, no negative were observed anymore for any combination.

Table 3: Germination behaviour and dry weight of cress seedlings exposed to an UW mix containing combinations of 3 active agents.

Substance*	Seedlings germinated with roots**			Dry weight**		
	+	0	-	+	0	-
EE2	3	2	1	1	5	0
E2	1	2	3	1	5	0
CZ	4	1	1	1	5	0
PI	2	3	1	1	5	0
IBU	2	1	3	2	4	0

* The full names of the substances are provided in Table 1.

** "+": germinate better with increasing concentrations of the active agents; "0": no effect or tendency observed; "-": germinate worse with increasing concentrations of the active agents

Based on the results it became obvious that the effect of the urine concentration in the UW mix had a larger effect on the germination behaviour than the pharmaceutical substances. Pre-tests of several cereals showed that the addition of the UW mix as control showed already so strong reactions that a further usage of these seed types was not possible within the experiment. This was also shown (although with a minor strong effect) for some of the cereals used. The germination rate of winter wheat decreased by 4 % and of winter barley by 17 %.

Table 4: Germination rates of investigated cereal types in water and a urine water mix (UW mix).

Liquid	Germination rate (%)			
	Winter wheat	Winter rye	Winter barley	Oat
Water	97	97	89	83
UW mix	93	97	74	97
Decrease of germination in UW mix compared to water	4	0	17	+16

Aside, it has to be pointed out that the timing of the specific trial within the overall time plan of the investigation (running for various months) had a major impact on the total growth of the seedlings. This can be shown nicely for cress which was investigated from February till May. The weight doubled: while the dry weight was 47 mg in February it had his peak in April with 107 mg (Figure 3).

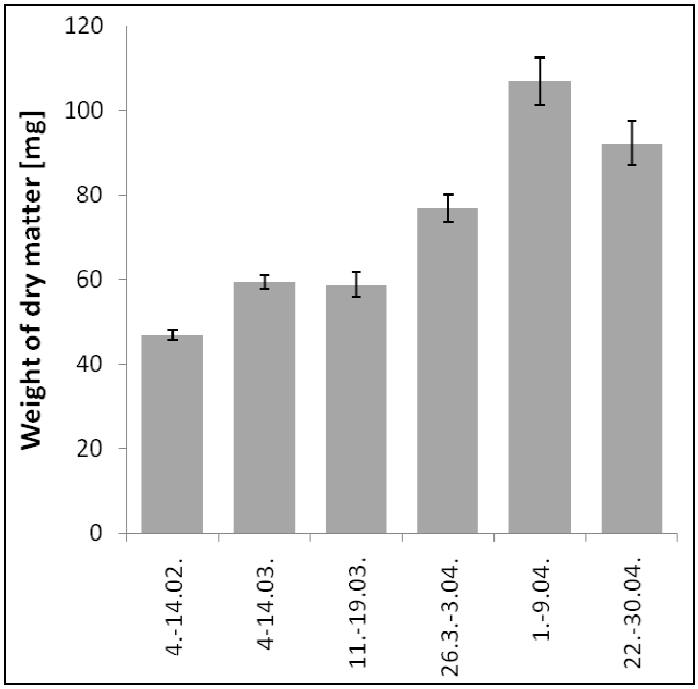

Figure 3: Average weight of dry matter of cress in the end of each trial. The graph reflects the weight increase of the cress seedlings used as control over time. The controls were seeded in the UW mix without addition of any pharmaceutical agent. Error bars show the standard deviation from the average among the 8 controls.

In the case of the trials with combinations of different pharmaceutical substances not all jars relevant for the same statistical evaluation could be run at the same time. Here, we standardised the results for the comparison via the control. Hence, we divided the actual weight determined in a jar by the average weight of the control jars (only UW mix) to allow comparison. This was based on the assumption that all jars run at the same time had the same surrounding conditions and that the control seedling run on UW mix are always exposed

towards the same basic stress and will react in the same of. Of course, this did not take into account the slight changes in the urine which obviously occurred. This is a clear weakness of the set-up.

Table 5: Change of seed weight by drying the seeds at 60°C for 100 h as well as over the time span of the experiment of 10 d.

Seed type	Seed weight (g)			Difference (%)	Seed weight (g)	Relation E/D (%)
	Original (O)	After drying (D)	Difference (O-D)		End of the experiment (E)	
Cress	0,049	0,046	0,0026	5,2	0,11	240
Winter wheat	0,98	0,89	0,088	9,0	0,68	77
Winter barley	1,1	1,0	0,090	8,3	0,88	88
Oat	0,74	0,67	0,075	10	0,57	85
Winter rye	0,60	0,55	0,050	8,3	0,44	80

Another constraint was that in the case of the cereals' seeds overall weight determined in the end of a test was lower than the weight of the seeds in the beginning (Table 5). The cereals' weight was 77 % (winter wheat) to 88 % (winter weight) their weight of the beginning. The only possible explanation assumed is the metabolism in the germination period and resulting use of the starch stored in the seed itself. Only cress, a fast growing organism, overcome this weight degrease within the time frame of the experiment and even showed a weight increase of 140 %. The slight weight reduction during the drying process supported this effect. Also here a larger reduction was observed for the cereals (8 to 10 %) then for cress (5.2 %).

CONCLUSION

Overall, it can be concluded that the potential effect of pharmaceutical substances contained in urine towards plants cannot be determined in germination experiments. The seedlings show sensitivity against pharmaceutical agents but not at the concentration levels the agents are expected for average German urine. Aside, the urine matrix itself is much more affecting the seedlings due to its specific matrix than the active agents. Also, in certain cases reactions of seedlings towards the pharmaceutical substances can be observed.

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