Two kinds of asymmetry in spring wheat leaf blade

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Abstract. The aim of the study was to find differences in the shape and asymmetry of the leaf blade of spring wheat grown at different dose of fertilizer. 52 landmarks were applied to the digitized images of the leaf blade and using the methods of geometric morphometrics the shape configuration was aligned. The components of the shape and asymmetry were estimated using Procrustes distances. Two types of asymmetry – fluctuating and directional were evaluated in Procrustes analysis of variance. The Procrustes distance showed a difference in the shape of the leaves of plants grown on mineral fertilizers in compare to the control (0.003; \( p < 0.001 \)). Visually, the width of the leaf blade of the plants formed with fertilization was bigger than in the control leaves. With an increase in the fertilizer dose, the Goodall criterion value increased from 30.0 to 46.2 (directional asymmetry; \( p < 0.0001 \)) and from 3.8 to 23.02 (fluctuating asymmetry; \( p < 0.0001 \)). There was no change in overall asymmetry of the experimental leaves, as Procrustean distance showed no difference between the centers of the sets in experimental variables (\( p ≥ 0.05 \)). Thus, an increase in the dose of mineral-organic fertilizer influenced both the shape and the asymmetry including the structure of the bilateral asymmetry of spring wheat leaf blades, influencing the homeostasis of plant development.

1. Introduction
The flag leaf is an important part of the cereal plant, which plays a large role in the formation of the ear grain. Recent studies have shown the morphology of the leaf, as well as the physiological and biochemical properties, depended on the genetic variety and geographical area [1].

Characteristics such as the flag-leaf angle, length and area of the flag leaf influenced the growth rate and productivity, showing different hereditary and genetic properties with the effects of dominance and epistasis [2, 3]. The interest to the bilateral asymmetry of leaf blades in cereals is also explained by the genetic nature of various types of asymmetry. Biometric genetics has been enriched with new advanced methods for assessing the genetic components of phenotypic variation and morphological variability. The Geometric morphometrics uses Procrustes distances to compare various shape configurations and even their components [4]. Directional asymmetry (DA) is known to be inherited; this type of asymmetry is close to the genetically determined component of variation.
Fluctuating asymmetry (FA) is considered a consequence of environmental influence, the epigenetic nature of this type of asymmetry is an intriguing field of study.

The separate testing of two types of asymmetry (DA and FA) was carried out using the methods of geometric morphometrics and genomic markers [5]. Methodically, not only Procrustes distances between the centers of sets are used, but also related methods, for example, the method of thin plate splines and the analysis of Procrustes residual differences [6]. When studying the properties of cultivated plants, various factors affecting productivity are studied. Recently, new methods of selection have been developed, including methods on the epigenetic level. In the proposed study, 2 types of flag leaf asymmetry were assessed among four populations of spring wheat grown at different doses of fertilizer. It is known the fluctuating asymmetry is a property of homeostasis that maintains a balance of biological system. Directional asymmetry interferes to the accurate evaluation of the FA value.

In the proposed paper, for a more detailed study, we used the coefficient of strength association two types of covariance matrices: symmetric and asymmetry. To evaluate the means of DA and FA, the Procrustes analysis of variance was used to find the F values of Goodall's criterion, taking into account the measurement error. The visualization of the shape elements was carried out on the basis of the canonical variate analysis (CVA). The working hypothesis meant the assumption an increase in fertilizer doses affects both the shape and the magnitude of the bilateral asymmetry in the leaf blade of spring wheat.

2. Materials and methods
Cultivation of spring wheat (Triticum aestivum L.) was carried out on gray forest medium loamy soil on the upland part of the flat relief of the Suzdal region of the Vladimir oblast (Russia). We used 4 doses of mineral fertilizer nitroammophos (NPK). The following doses were input according to the accepted technology: 1) intensive (N90P90K90), 2) highly intensive (N120P120K120), 3) intensive organo-mineral (60t organic fertilizer + N90P90K90) and: 4) highly intensive organo-mineral (80t organic fertilizer + N120P120K120). From each plot with an area of 7 × 20 m, 25 flag leaves with a length of 12 to 16 cm were randomly selected at the stage of wax ripeness in the third decade of June 2020. Protective lines 10 × 7 m, separated by furrows from the experimental sites, served as a control. There were in total three control lines that were evenly spaced between the four experimental sites.

The leaves were stored in 95% ethanol and, no later than 2-4 days after harvesting, were dried in air and attached with PVA glue on the paper by abaxial surface up. The leaf blade served as an experimental unit: they were scanned twice with a Canon MF3010 at a resolution 300 dpi, the landmarks (LMs) were also applied twice on each image.

First, two landmarks were placed at the base and at the apex of the flag leaf. They played role of middle axis. Then 50 LMs were applied along the contour of the blade. Thus, two LMs classified as LMs of type 1, the other 50 were semilandmarks. The last ones applied in a regular order had XY coordinates and had homologous properties, so their coordinates could be compared to find the asymmetry (DA and FA). Each file was saved in TPS format, and then combined file was formed, which was used in the MorphoJ for create a data set. Thus, 4 datasets were created, one for each soil fertilization and three datasets for control plants. Visually, the leaves were curved, especially in the distal and proximal parts, the middle part of the leaf blade visually varied less.

The statistical significance of DA and FA was tested in Procrustes analysis of variance. The interaction of the mean squares of the factor “side” and “leaf” indicated a deviation in fluctuating asymmetry in the leaf blade, and, accordingly, in the developmental stability deviation. The factor “side” indicated the presence of directional asymmetry. Numerically FA, as well as directional asymmetry, was evaluated by the Goodall F criterion (F1,5 and F5). The difference in the shape was defined by the magnitude of the consensus i.e. the averaged shape in the morphospace with a value equal to the square root of the sum of the squares of the coordinates LMs along the X- and Y axes.

The symmetrical component of the shape was defined by the variation of LMs among the samples and served to characterize the shape in term of Procrustes distance. The asymmetric component of the
shape was determined by the variation of LMs on the left and right sides, signifying the overall asymmetry of the shape, which included both fluctuating asymmetry and directional asymmetry. The canonical covariance analysis of the symmetry and asymmetry matrices served as additional research tools. It was performed using the XY coordinate dataset. The distance between the centers of the sets of coordinates of the first principal component of the variation was assessed. The coefficient of the strength of the association between the symmetric and asymmetric components of the shape (RV) was computed for comparing covariance matrices. The proceedings LMs coordinates was fulfilled in TPS package (Rohlf, 2017-2019).

All statistical analyzes and visualization were carried out in the MorphoJ 1.06d media (Klingenberg, 2011) using a 95% significance level; permuting of samples with n = 10 000 replicates was used, which reduced the risk of antisymmetry presence in samples.

3. Results and discussion

3.1 Shape of blade
First we assessed the overall asymmetry. In geometric morphometrics it assumes the testing asymmetry of the shape. To visually assess the difference in the shape of the leaf, a covariance analysis was performed. The first component variation decomposition occupied a large portion c. 90% overall variance and was used for comparison.

The symmetrical component of the shape was compared to the average symmetric consensus. Figure 1 shows the difference between the shapes of the leaves of plants grown with fertilization from the consensus of the control sample.

![Figure 1](image1.png)

Figure 1. Comparison of experimental leaves (blue) versus control consensus (cyan).

Thus, about 90% of the experimental leaf blades were wider than the control. The quantitative evaluation was provided using Procrustes distances between the centers of the coordinate sets. It was 0.003 (p <0.0002). Decomposition of sets of values in two-dimensional space into two components of variation showed two ranges of values (figure 2A).

![Figure 2A](image2A.png)
![Figure 2B](image2B.png)

Figure 2. Visualization of CVA results. A: Decomposition two components of the variation CV1 and CV2 in two-dimensional space. B: the frequency diagram of the first component variation. Cyan color - experiment, red color - control.
Differences were also found in the frequency diagram of the first components of the variation (figure 2B). The increase in leaf width in experimental plants was expected. To determine the variability in asymmetry, the asymmetry component as part of the overall shape of the leaf blade was assessed.

### 3.2 Bilateral asymmetry

The asymmetric component showed a shift of coordinates to one side, indicating a directional asymmetry (figure 3).

**Figure 3.** Asymmetric component of the experimental sample (blue) and control (cyan). Downward shift meant right-sided directional asymmetry of paired landmarks (№41-14, 42-13, 43-12, 44-11, 45-10 46-9 47-8 and 48-7).

The asymmetric component differed in the two samples. The Procrustes distance had the same value as in the symmetric components – 0.003; *p* < 0.0003. This gave the right to talk about the conjugation in the difference in shape and in overall asymmetry as both categories changed parallel.

The images of the leaves were carried out abaxial side up; therefore, we could conclude on the directional asymmetry to the left half of the leaf blade, if we normally view the adaxial (glossy) side. Possibly the left-sided DA caused by the accumulation of auxin in the left part of the leaf bud. The quantitative testing of asymmetry was performed in Procrustes analysis of variance (table 1).

**Table 1.** Comparison of leaf blade asymmetry in GPA.

| Effect         | % of total MS | df   | F     | % of total MS | df   | F     |
|----------------|---------------|------|-------|---------------|------|-------|
| Leaf           | 0.7           | 9950 | 0.97* | 2.4           | 7450 | 0.85* |
| Side           | 98.4          | 50   | 129.9*** | 94.6         | 50   | 33.27*** |
| Leaf×side      | 0.8           | 9950 | 9.78*** | 2.8           | 7450 | 27.78*** |
| Residual       | 0.1           | 20000| 1.71×10⁻⁶ | 0.1           | 15000| -     |

Notes: df = degree of freedom; MS = mean square; F = Goodall criterion; *** – *p* < 0.0001; ns – not significant.

Leaf blades did not differ among the samples (“leaf” was not statistically significant), the value of directional asymmetry (“side”) exceeded FA (“leaf × side”) up to 10 times in the experimental group. In the control group, both types of asymmetry were almost the same. The measurement error in the experiment was 10.2% of the “leaf × side” MS value and 3.6% in the control. We explain the high directional asymmetry in the experimental group by the influence of fertilizers that stimulate the uneven growth of the blade in the left half. The asymmetry in the control was weaker, but a significant part (more than 50%) was occupied by fluctuating asymmetry. GPA was carried out for each dose sample (table 2).

**Table 2.** Effect of different doses (increasing value from dose № 1 to № 4) on the bilaterally symmetrical properties of the leaf blade *Triticum aestivum* L.

| Dose | Effect       | SS   | MS     | df   | F     |
|------|--------------|------|--------|------|-------|
| 1    | Leaf         | 0.016| 6.34×10⁻⁶ | 2450 | 0.97* |
|      | Side         | 0.010| 0.0002 | 50   | 30*** |
|      | Leaf × side  | 0.016| 6.56×10⁻⁶ | 2450 | 3.83*** |
|      | Residual     | 0.009| 1.71×10⁻⁶ | 5000 | -     |
The individual variability of the leaf blade varied: doses №1, 2 and 4 showed no difference in shape, dose №3 did not. FA value increased from dose №1 (F_{I\times S} = 3.83) to dose №4 (F_{I\times S} = 23.92). Directional asymmetry also increased, although both dependencies were at the same level of probability $p = 0.0001$. Directional asymmetry showed a higher value of the F criterion than fluctuating asymmetry. We explain the increase in directional asymmetry by a more intensive growth rate of the leaf blade with uneven and directed cell division in the left half of the blade. Homeostasis in this case deviated in the direction of increasing DA. The sum of the values MS factor “side” and “ind × side” increased. The overall asymmetry could be analyzed visually and estimated in Procrustes distances. The first components of the data sets were not separated visually and the Procrustes distances showed no difference ($p \geq 0.05$; table 3).

**Table 3.** Procrustes distances among four dose groups and $p$-values from permutation tests.

| Procrustes distances | $p$-values |
|----------------------|------------|
|          | 1dose | 2dose | 3dose | 2dose | 3dose |
| 2dose    | 0.002 |       |       | 0.34  |       |
| 3dose    | 0.003 | 0.004 |       | 0.13  | 0.038 |
| 4dose    | 0.003 | 0.004 | 0.003 | 0.039 | 0.03  | 0.05  |

As can be seen from the table, there was no statistical difference in asymmetry at different doses, but the structure of the asymmetry was different: FA increased with increasing dose; in the control leaf pool, the $F_{I\times S}$ was much higher than $F_S$ (see table 1).

Partial least squares (PLS) regression was performed for symmetry covariance matrices. Two data were compared: the symmetrical component and the asymmetric component of the shape variation. The RV coefficient, showing the strength of the association between the two blocks, was higher in the pool of experimental leaves (RV = 0.09; $p < 0.0001$) than in the control (RV = 0.05; $p = 0.01$). We explain this by the greater variation in leaves in the experimental group, both in their asymmetry and in the shape. The RV coefficient was also obtained in experimental samples. The highest strength association was obtained for dose №4 (0.3; $p < 0.0001$). Other doses showed a probability level $p = 0.0002$ and RV = 0.14–0.2 which corresponded to the assumption of a relationship between the shape and asymmetry.

4. **Conclusion**

The previous studies carried out on winter wheat showed a mixture of DA and FA in the same ratio, which did not depend on the fertilizer dose [7]. When working with rye plants, two doses of the mineral fertilizer NPK were tested. At a low dose, a “pure” FA was obtained, devoid of an admixture of directional asymmetry, while a high dose showed a mixture of both types of asymmetry [8].
In the present study, only an increase in FA (ind × side) was obtained along increasing fertilizer dose. The strength of the association (correlation between the two matrices) also increased.

At each dose, FA and DA had the same statistical significance at the same level of probability \( p \), which did not allow us to assert a statistically significant dependence dose – asymmetry.

The findings are consistent with those obtained using the leaf width trait of winter wheat in 2018. Such doses of fertilizer caused an increase in the FA value with an increase in the dose of the applied fertilizer [9].

Directional asymmetry prevailed in the plants grown on fertilizers in \( F_s \) values. We explain this by the influence of mineral and organic fertilizers on the uneven growth of the plate, which reflected the genetic predisposition of wheat to maintain homeostasis. The different responses of rye and wheat plants could well be explained by genetic differences in species.

Thus, the hypothesis about the effect of applied fertilizer NPK on the variability of the shape and general asymmetry of leaf blades of spring wheat was confirmed. The subsequent studies are planned to be carried out focusing on the heterogeneity of the asymmetry distribution and some morphological characteristics of the flag leaf.

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